



11/25/97

A

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Anticipated Classification
of this Application:

Class _____ Subclass _____

Prior Application:

Exam'r: Remy Yucel

Group Art Unit: 1805

Attorney's

Docket No.:

Enz-53(C)

FILED BY EXPRESS MAIL

Hon. Commissioner of Patents and Trademarks
Washington, D.C. 20231
BOX FWC (if under 1.62)

COPY

Sir:

This is a request for filing a X CONTINUATION DIVISIONAL
 CONTINUATION-IN-PART application under 37 CFR X 1.60
37 CFR 1.62, of pending prior application Serial No. 08/574,443
filed on December 15, 1995 of Elazar Rabbani et al.
Inventor(s)

for:

NOVEL PROPERTY EFFECTING AND/OR PROPERTY EXHIBITING COMPOSITIONS FOR THERAPEUTIC AND DIAGNOSTIC USES

Title of Invention

1. X Enclosed is a copy of the prior application, as originally filed and an affidavit or declaration verifying it as a true copy.
2. X A copy of the verified statement under 37 CFR 1.9 and 1.27 establishing small entity status in the prior application is enclosed. Small entity status is still applicable.
3. X The filing fee is calculated as follows:

CLAIMS AS FILED, LESS ANY CLAIMS CANCELLED BY AMENDMENT									
	Number Filed		Number Extra		RATE			FEE	
					Small Entity	Other Entity		Small Entity	Other Entity
Total Claims	1	=	0	x	\$ 11	\$ 22	=	\$ 395	\$ ---
Independent Claims	1	=	0	x	\$ 41	\$ 22	=	\$ 0	\$ ---
Multiple Dependent Claims Presented: <u> </u> Yes <u>x</u> No					\$ 135	\$ 270		\$ ---	\$ ---
* If the difference in Col. 1 is less than zero, enter "0" in Col.2						BASIC FEE		\$ 395	\$ ---
						TOTAL FEE		\$ 395	\$ ---

Attorney's Docket No. Enz-53(C)

EXPRESS MAIL CERTIFICATE	
"Express Mail" Label No.	EM449423938
Deposit Date	NOVEMBER 25, 1997
I hereby certify that this paper and the attachments herein are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Commissioner of Patents and Trademarks, Washington DC 20231	
<i>Ronald C. Fedus</i>	NOV 25 97
Ronald C. Fedus Reg. No. 32567	Date

08978632 112597

Elazar Rabbani et al.

Serial No.: 08/574,443

Filed: Herewith

Page: 2 (Request for a Continuation Application Under 37 C.F.R. 1.60 -
November 25, 1997)

4. X The Commissioner is hereby authorized to charge payment of the filing fee as well as the following fees associated with this application or to credit any overpayment to Deposit Account No. 05-1135. A **duplicate** copy of this sheet is enclosed.
- X Any additional filing fees required under 37 CFR 1.16.
- X Any patent application processing fees under 37 CFR 1.17.
5. X The Commissioner is hereby authorized to charge payment of the following fees during the pendency of this application or credit any overpayment to Deposit Account No. 05-1135. A **duplicate** copy of this sheet is enclosed.
- X Any patent application processing fees under 37 CFR 1.17.
- The issue fee set in 37 CFR 1.18 at or before mailing of the Notice of Allowance, pursuant to 37 CFR 1.311(b).
- Any filing fees under 37 CFR 1.16 for presentation of extra claims.
6. A check in the amount of \$ is enclosed.
7. X Cancel claims 25-244.
8. Amend the specification by inserting before the first line of the sentence: --- This is a continuation, X divisional, continuation-in-part, of application Serial No. 08/574,443, filed on December 15, 1995. ---
9. Transfer the drawings from the prior application.
10. X Informal X Formal drawings are enclosed.
11. Abandon said prior application as of the filing date accorded this application. A **duplicate** copy of this sheet is enclosed for filing in the prior application file.
12. Priority of application Serial No. filed on is claimed under 35 U.S.C. 119.
The certified copy of the priority application has been filed in prior application Serial No. , filed .
13. The prior application is assigned of record to Enzo Diagnostics, Inc., c/o Enzo Biochem, Inc., 527 Madison Avenue, New York, New York 10022. [A copy of the originally submitted Assignment and the Notice of Recordation are enclosed herewith.]
14. A preliminary amendment is enclosed.

Elazar Rabbani et al.

Serial No.: 08/574,443

Filed: Herewith

Page: 3 (Request for a Continuation Application Under 37 C.F.R. 1.60 -
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15. X The power of attorney in the prior application is to:
Ronald C. Fedus, Reg. No. 32,567, and John J. Santalone,
Reg No. 32, 794.
- (a) X The power appears in the original papers in the prior
application.
- (b) Since the power does not appear in the original
papers, a copy of the power in the prior application is
enclosed.
- (c) A copy of a new substitute power of attorney is
enclosed.
- (d) Address all future communications to: (May only be
completed by applicant, or attorney or agent of
record.)
- Ronald C. Fedus, Esq.
Enzo Diagnostics, Inc.
c/o Enzo Biochem, Inc.
527 madison Avenue (9th Floor)
New York, New York 10022
16. X Also accompanying this Request is a Petition to Revive An
Unintentionally Abandoned Application Under 37 C.F.R.
1.137(b).
17. X I hereby verify that the attached papers are a true copy of prior
application Serial No. 08/574,443.
18. The parent application (Serial No. 08/574,443) was
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will be copending with the filing of this new continuation application.

The undersigned declares further that all statements made herein of his own knowledge
are true and that all statements made on information and belief are believed to be true;
and further that these statements were made with the knowledge that willful false
statements and the like so made are punishable by fine
or imprisonment, or both, under Section 1001 of Title 18 of the United States

Elazar Rabbani et al.

Serial No.: 08/574,443

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Page: 4 (Request for a Continuation Application Under 37 C.F.R. 1.60 -
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application or any patent issuing thereon.

NOV 25 97
Date


Signature [Ronald C. Fedus]

Registration No. 32, 567

Attorney for Applicants

☐ Inventor(s)

☐ Assignee of Complete Interest

☒ Attorney or Agent of Record

☐ Filed under 37 CFR 1.34(a)

Address of Signator:

Enzo Diagnostics, Inc.

c/o Enzo Biochem, Inc.

527 madison Avenue (9th Floor)

New York, New York 10022

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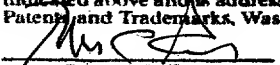
**NOVEL PROPERTY EFFECTING AND/OR PROPERTY EXHIBITING
COMPOSITIONS FOR THERAPEUTIC AND DIAGNOSTIC USES**

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 Ronald C. Fedus Reg. No. 32567	<u>NOV 25 97</u> Date

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Page: 3 (Request for a Continuation Application Under 37 C.F.R. 1.60 -
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- (d) Address all future communications to: (May only be
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Ronald C. Fedus, Esq.
Enzo Diagnostics, Inc.
c/o Enzo Biochem, Inc.
527 madison Avenue (9th Floor)
New York, New York 10022

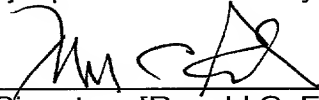
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Page: 4 (Request for a Continuation Application Under 37 C.F.R. 1.60 -
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Code and that such willful false statements may jeopardize the validity of the
application or any patent issuing thereon.

NOV 25 97
Date


Signature [Ronald C. Fedus]
Registration No. 32, 567
Attorney for Applicants
☐ Inventor(s)
☐ Assignee of Complete Interest
☒ Attorney or Agent of Record
☐ Filed under 37 CFR '1.34(a)

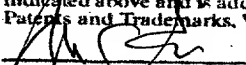
Address of Signator:
Enzo Diagnostics, Inc.
c/o Enzo Biochem, Inc.
527 madison Avenue (9th Floor)
New York, New York 10022

APPLICATION
FOR
UNITED STATES LETTERS PATENT

To all Whom it may concern:

Be it known that

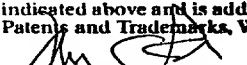
Elazar Rabbani,
Jannis G. Stavrianopoulos,
James J. Donegan,
Dakai Liu,
Norman E. Kelker, and
Dean L. Engelhardt

EXPRESS MAIL CERTIFICATE	
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 Ronald C. Fedus Reg. No. 32,567	NOV 25 97 Date

have invented certain new and useful improvements in

NOVEL PROPERTY EFFECTING AND/OR PROPERTY EXHIBITING
COMPOSITIONS FOR THERAPEUTIC AND DIAGNOSTIC USES

of which the following is a full, clear and exact description.

EXPRESS MAIL CERTIFICATE	
"Express Mail" Label No.	EF436871946
Deposit Date	DECEMBER 15, 1995
I hereby certify that this paper and the attachments herein are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Commissioner of Patents and Trademarks, Washington DC 20231	
 Ronald C. Fedus Reg. No. 32,567	DEC 15 1995 Date

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Honorable Commissioner of
Patents and Trademarks
Washington, DC 20231

Box: PATENT APPLICATION

Sir:

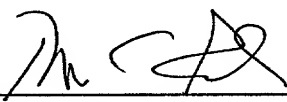
Transmitted herewith for filling is the patent application of:

Inventor(s): Elazar Rabbani,
Jannis G. Stavrianopoulos,
James J. Donegan,
Dakai Liu,
Norman E. Kelker, and
Dean L. Engelhardt

For: **NOVEL PROPERTY EFFECTING AND/OR PROPERTY
EXHIBITING COMPOSITIONS FOR THERAPEUTIC &
DIAGNOSTIC USES**

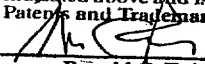
Respectfully submitted,

DECEMBER 15, 1995
Date



Ronald C. Fedus
Registration No. 32,567
Attorney for Applicants

ENZO THERAPEUTICS, INC.
ENZO DIAGNOSTICS, INC.
c/o Enzo Biochem, Inc.
575 Fifth Avenue (18th Floor)
New York, New York 10017
Telephone (212) 856-0876

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 Ronald C. Fedus Reg. No. 32,567	DEC 15 1995 Date

NOVEL PROPERTY EFFECTING AND/OR PROPERTY EXHIBITING COMPOSITIONS FOR THERAPEUTIC AND DIAGNOSTIC USES

FIELD OF THE INVENTION

This invention relates to compositions including nucleic acid constructs, conjugates, and vectors which are capable of effecting and exhibiting biological function within a cell or cell containing biological system.

All patents, patent publications, scientific articles cited or identified in this application are hereby incorporated by reference in their entirety in order to describe more fully the state of the art to which the present invention pertains.

BACKGROUND OF THE INVENTION

An alternative to viral mediated gene delivery is direct delivery of nucleic acid. This approach has several limitations including low efficiency of transfer, low stability and lack of cell specificity. In order to overcome some of these limitations other approaches have been made. These include non-specific ionic complexes with polycations such as polylysine (Wu and Wu, US Patent No. 5,166,320, contents of which are incorporated herein by reference) and histone. These bind non-specifically with the nucleic acid construct through polycations or basic proteins, such as histones. However, the resulting complexes still suffer some limitations including lack of

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uniformity of the complexes, lack of specificity with respect to polycation binding to specific regions of the nucleic acid construct, potential interference of complexes with nucleic acid and possible untimely disassociation of the complex or lack of timely disassociation of the complex leading to a lack of stability of these nucleic acid polycation or nucleic polypeptide complexes.

Nucleic acid transfer to cells can take place by various methods.

Such methods can utilize free nucleic acid, nucleic acid constructs or nucleic acid as part of the genome of a virus or bacteriophage vector.

Wu et al., US Patent No. 5,166,320, utilized a polynucleotide in a nonspecific association with the polycation polylysine. These complexes suffer limitations including lack of consistency of composition, lack of specificity with respect to polycation binding to specific regions of the nucleic acid construct, potential interference of complexes with nucleic acid and possible untimely disassociation of the complex or lack of timely disassociation of the complex leading to a lack of stability of these nucleic acid or histone polycation or nucleic polypeptide complexes. This procedure does not provide for delivery of virus vectors. Furthermore, cell transformation efficiencies are still low.

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Methods for retrovirus mediated gene transfer to hematopoietic cells ex vivo has been attempted in the presence of fibronectin or fibronectin fragments. Fibronectin binds to retroviruses but not to any other viruses, nucleic acids or nucleic acid constructs. Williams and Patel, WO 95/26200 (the contents of which are incorporated herein by reference), have transformed hematopoietic cells with retroviruses in the presence of fibronectin. The use of fibronectin in this way is limited only to use with some retrovirus vectors and not with other virus vectors or with nucleic acids.

It is desirable to form multimeric complexes for two primary reasons. The formation of such complexes results in an additive effect such that one can obtain collective activity of the monomeric units within a complex or these complexes could provide enhanced binding properties compared to the individual compounds or monomeric units, either through cooperative binding effects or through neighboring effects which produce higher localized concentrations. Polyligands usually exhibit higher binding affinities in the polymeric form than in the monomeric form as seen by the binding of polynucleotide sequences to their complementary sequences when compared to the binding of the monomeric units.

Multimeric complexes have been formed either by crosslinking of monomeric compounds directly or through a matrix or through the formation of noncovalent linkages. Examples of multimeric complexes formed by the

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crosslinking of a given compound such as enzymes, either directly or through a matrix are described in US Patent No. 4,687,732 (contents of which are incorporated herein by reference), whereby a visualization polymer composed of multiple units of a visualization monomer is linked together covalently by coupling agents which bond to chemical groups of the monomer. Examples of multimeric complexes made through the formation of noncovalent linkages such as ligand-receptor systems are the PAP (peroxidase-anti-peroxidase) complexes and APAAP (alkaline phosphatase-anti-alkaline phosphatase) complexes in common use as immunological reagents and the streptavidin-biotinylated enzyme complexes used for detection of biotinylated entities.

In the case of complexes formed by crosslinking or noncovalent binding, there are limitations with respect to the spacing and the chemical milieu of the monomeric unit within the complex which may affect the function and activity of the monomeric unit and as the size of the complex grows, solubility may be affected.

Efforts to regulate expression of procaryotic genes by eucaryotic processes have been attempted by Schwartz et al. (1993 Gene 127: 233) (also incorporated herein by reference) who introduced an intron sequence from a eucaryotic gene into a procaryotic gene. However, when introduced into a cell capable of mRNA processing, the gene expressed an altered protein in

which additional amino acids were present due to the presence of flanking exon sequences associated with the inserted intron. This limitation is inherent in this approach since this method of intron isolation requires the a priori presence of inherent restriction sites in the exon regions flanking the intron, and intron insertion requires the presence of appropriate restriction sites in the gene receiving the intron. Therefore, even after the excision of the intron from the RNA, the flanking exon sequences remain as part of the coding sequence of the mature RNA molecules. Furthermore, the number of sites for intron insertion on the receiving gene is severely limited by the availability of appropriate restriction sites.

The alteration of the gene product by this approach may have unpredictable effects on the function of the gene product and severely limits the applicability of this method to specific instances. In the example of Schwartz et al. the additional amino acids had no apparent effect on the activity of the protein synthesized in the capable cell, but this is not always a predictable quality since it depends upon the site where the additional amino acids are incorporated. For instance, a short sequence coding for a small peptide introduced into the amino end of T7 RNA polymerase by Dunn et al. (1988 Gene 68: 259) (also incorporated herein by reference) had no apparent effect on enzyme activity. However introduction of the same sequence into a site near the carboxy terminus resulted in nearly complete loss of enzyme activity. Thus, the incorporation of extra amino acids as a

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result of introducing an exon into a coding sequence by the method of Schwartz et al. could have a drastic mutagenic effect.

Systems derived from procaryotic elements can produce functional products in mammalian cells. T7 RNA polymerase, an enzyme derived from an *E. coli* bacteriophage, has been expressed both transiently and stably in mammalian systems (Fuerst et al., 1986 Proc. Nat. Acad. Sci. U.S.A. 83: 8122, the contents of which are herein incorporated by reference). When synthesized in a mammalian environment, it is capable of acting upon genes under the control of a T7 promoter to produce transcripts that can be translated to provide a functional gene product. Large amounts of RNA can be transcribed from the T7 promoter (comprising up to 30,000 RNA molecules per cell, Lieber et al. 1993, also incorporated by reference).

In eucaryotic systems success has only been achieved by the use of a binary system with the polymerase on one construct and the T7 promoter on a separate construct, In this way either sequential transfections (Lieber et al. 1989 Nucleic Acids Res 17, 8485) (also incorporated by reference) or co-transfections with separate plasmids (Lieber et al. 1993 Methods Enzym. 217, 47) (incorporated by reference) or transfection with a plasmid containing a T7 promoter followed by infection with a recombinant vaccinia virus coding for T7 RNA polymerase (Fuerst et al. 1986 Proc. Nat. Acad. Sci. U.S.A. 83, 8122) must be done. Since T7 RNA polymerase can be

cloned only free of a T7 promoter sequence (Davenloo et al. 1984 Proc. Nat. Acad. Sci. U.S.A. 81: 2035) (incorporated herein by reference), it appears that attempts to clone both elements in a single construct fail due to an event where synthesis of the T7 RNA polymerase-initiated transcription from the downstream promoter continues around the plasmid to direct more synthesis of T7 RNA polymerase leading to a cytotoxic autocatalytic cascade. A similar strategy of elimination of cognate promoters has been described for the cloning of the bacteriophage T3 (Morris et al. 1986 Gene 41: 193) and SP6 (Kotani et al. 1987 Nucl. Acids Res. 15: 2653) (both publications incorporated herein by reference) RNA polymerases. However, compatibility of these elements has been achieved by the addition of two modifications to the construct, *i.e.*, inhibition of the T7 RNA polymerase by the presence of T7 lysozyme and the use of a repressible T7 lac promoter (Dubendorff and Studier 1991 J. Mol. Biol. 219: 61, 1991, incorporated herein by reference). Both of these limitations are required in order to obtain a construct.

The introduction of genetic material into cells can be done by two methods. One method is the exogenous application of nucleic acids which act directly on cellular processes but which themselves are unable to replicate or produce any nucleic acid. The intracellular concentrations of these molecules that must be achieved in order to affect cellular processes is dependent on the exogenous supply. Another method for nucleic acid delivery is the

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Parameter	Value	Unit
Initial concentration	1.0	mol/L
Temperature	25	°C
Time	0	min
Time	10	min
Time	20	min
Time	30	min
Time	40	min
Time	50	min
Time	60	min
Time	70	min
Time	80	min
Time	90	min
Time	100	min
Time	110	min
Time	120	min
Time	130	min
Time	140	min
Time	150	min
Time	160	min
Time	170	min
Time	180	min
Time	190	min
Time	200	min
Time	210	min
Time	220	min
Time	230	min
Time	240	min
Time	250	min
Time	260	min
Time	270	min
Time	280	min
Time	290	min
Time	300	min
Time	310	min
Time	320	min
Time	330	min
Time	340	min
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Time	380	min
Time	390	min
Time	400	min
Time	410	min
Time	420	min
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Time	960	min
Time	970	min
Time	980	min
Time	990	min
Time	1000	min

The effectiveness of antisense therapy depends in large part on three major factors: a) the rate of transcription of antisense RNA, b) the cellular location of the RNA and c) the stability of the RNA molecules. While previous studies have addressed each of these factors, all three have not

been addressed in a single approach. The present invention utilizes AS sequences substituted for nucleotide sequences in the U1 and other hnRNAs to achieve high nuclear concentrations of stable antisense RNA sequences.

U1, U2 and other snRNAs are nuclear-localized RNA molecules complexed with protein molecules. (Dahlberg and Lund 1988 in Structure and Function of Major and Minor Small Nuclear Ribonucleoprotein Particles, M. Birnstiel, Ed., Springer Verlag, Heidelberg, p38: , Zieve and Sautereau 1990 Biochemistry and Molecular Biology 25;1, all of which are incorporated herein by reference).

The various promoters for U1, U2 and other snRNA operons are very strong and produce large amounts of RNA. U1 and other snRNAs have signals for export to the cytoplasm where specific proteins are complexed before reimportation to the cytoplasm as snRNPs (Figure 41). snRNAs are very stable molecules. They form very highly ordered stem and loop structures (Figure 43) which, when complexed with specific proteins, form snRNP, or spliceosomes.

Antisense and other nucleic acid molecules which affect gene expression by acting on and altering RNA transcripts can derive certain advantages by confinement to the nucleus. Higher concentrations can be maintained in the smaller volume of the nucleus, interactions with target RNA can occur prior

to their being used for expression and there would be no competition with messenger binding ribosomes.

Addition of antisense sequence to U2 RNA (Izant and Sardelli 1988 in Current Communications in Molecular Biology, Cold Spring Harbor, p141, incorporated herein by reference) as a means of delivering antisense sequences altered the properties of normal U2 transcripts. Hybrid U2 molecules formed by insertion of antisense sequences into a restriction site in the 5' end of the U2 transcript region showed decreasing antisense effectiveness with increasing insert size. Inserts longer than 250 bases substantially reduced antisense effectiveness. Furthermore, hybrids did not accumulate in the nucleus as efficiently as their wild type counterparts with the fraction of hybrids in the nucleus decreasing as insert length increased.

Yu and Weiner (1988 in Current Communications in Molecular Biology, Cold Spring Harbor, p141, contents incorporated by reference) substituted 9 base antisense sequences directed at target sequences surrounding splice sites in mRNA. The antisense substitutions were made at the 5' end of U1 RNA. None of the antisense substitutions affected the level of targeted species of mature cytoplasmic RNA.

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Constructs have been designed to increase antisense effectiveness by the inclusion of more than one targeting element in a single transcriptional unit. Multivalent constructs prepared in this way can produce numerous target directed entities acting on multiple target sites in nucleic acids. (Chen et al., 1992 in Antisense Strategies, Annals of the New York Academy of Sciences 660;271; Zhou et al. Gene 1994 149;33, both publications incorporated herein by reference). Different approaches to inhibition can be incorporated into a multivalent transcript as shown by Lisiewicz et al. (1993 Proc Natl Acad Sci USA 90, 8000, also incorporated by reference) who combined multiple copies of the HIV TAR with an antisense sequence to HIV gag on the same transcript.

The use of multivalent targeting by the inclusion of more than one targeting element on the same transcript provides a method for counteracting the high mutation rate of viruses such as HIV due to the unlikely event of simultaneous mutation of multiple target sequences. However, the common means of accomplishing these designs is the inclusion of the product entities on a single transcript. This approach suffers from the following limitations:

- a) The total number of RNA molecules available as effective entities is limited by the strength of the single promoter;

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b) During stable transformation of a cell, the integration event can disrupt the nucleic acid template sequence responsible for expression of the antisense sequence;

c) The use of multivalent transcripts is not favorable when one product entity present on the transcript acts on targets present in one cellular locale and another product entity present on the same multivalent transcript acts on targets present in a different cellular locale. This was the approach reported by Lisziewicz et al. (1993) where multiple TAR sequences, which act to bind the HIV tat protein in the cytoplasm, were present on the same transcript with antisense sequences for the HIV gag RNA, which are most effective in the nucleus.

Although there have been major efforts to find effective antiviral treatments, at the present time the only success has been in a diminution of virus growth rather than elimination of the virus. Among the efforts that have been pursued are attempts to prevent initiation of the virus replication cycle by preventing the virus from entering the cell by immunization or by treatment with antibodies or with proteins that interfere with virus recognition of a cell by interacting with the virus or the virus receptor site on the cell. These include unsuccessful treatment with high levels of soluble CD4 (Husson et al., 1992, incorporated by reference). In addition, efforts have been made to combat HIV infection after virus entry into a cell using protease inhibitors for

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preventing processing of viral polypeptides into functional proteins and varied nucleoside analogues which can block replication of the virus by inhibiting the activity of the virally encoded reverse transcriptase and other functions necessary for virus propagation. Stages of the processes of viral infection and viral replication cycle have been examined for the possibility of pharmacological or immunological intervention of the disease process. However, as independent and effective therapeutic agents, both immunological and small molecule inhibitors have failed to stem the progression of AIDS, and major problems remain in terms of effectiveness and the rise of viruses resistant to small molecule therapeutic agents. Even the application of combinations of immunological and small molecule agents has not been successful.

The introduction of genetic information into cells either to replace a function or to introduce a new function has provided an effective means for the treatment of viral infection. Genetic therapy approaches have been used to impart cell resistance to viruses by mechanisms which act intracellularly on the viral replication process (see Yu et al., Gene Therapy 1, 13-16 [1994, incorporated by reference). A result of these studies is that, *in vitro*, the effectiveness of genetic therapies is sensitive to virus concentration. Experiments *in vitro* that showed substantial levels of resistance at low ratios of virus to cells, at higher ratios showed a "breakthrough" phenomenon characterized by a period of seeming effectiveness followed by a surge in the

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virus production (Sczakiel et al. 1992 J. Virol **66**;5576 :Scakiel and Pawlita 1990 J. Virol. **65**;468, all of which are incorporated by reference). Thus *in vitro*, at lower virus:cell ratios some genetically treated cells demonstrate longer survival times that at higher virus:cell ratios.

Compartmentalization of function is critical to regulated processes in eucaryotic cells. For example, the major part of cellular DNA is organized into chromosomes located in the nucleus where transcription of genetic information takes place. The major part of RNA synthesized in the nucleus is transported to the cytoplasm where it is translated. Other subcellular compartments for localized function include the Golgi apparatus, endoplasmic reticulum, nucleolus, mitochondria, chloroplast and the cellular membrane. Thus, a variety of mechanisms exist either to retain macromolecules in specific cellular compartments or to transport macromolecules from one cellular compartment to another. For example, in the directed exit of mature mRNA out of the nucleus into the cytoplasm, the presence of a 5' cap, removal of introns and addition of a poly A sequence are all believed to contribute to the signal that directs the relocation (reference).

Some RNAs, such as small nuclear RNAs (snRNAs) involved in spliceosome assembly, are relocated by sequential transportation (Dahlberg and Lund, 1988, in Structure and Function of Major and Minor Small Ribonuclear Particles, M. Birnstiel, ed., Springer Verlag, Heidelberg, pg. 38, incorporated

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by reference). After transcription in the nucleus, the presence of the 5' cap and the processed 3' terminus generate a bipartite signal for transport of U1 RNA into the cytoplasm. At this point there is further processing of the RNA by excision of a few nucleotides and hypermethylation of the 5' cap. The binding of spliceosome proteins present in the cytoplasm to the Sm region of the U1 RNA in combination with the hypermethylation is believed to generate a signal for the reimportation of the RNA back into the nucleus.

In contrast to most mRNA, most proteins do not need to be transported from their site of synthesis in the cytoplasm. However, some proteins that function in transcription, replication or other nuclear maintenance functions need to be present in the nucleus to function properly. In this case a polypeptide signal sequence present in the protein directs the transport of the protein from the cytoplasm into the nucleus. Still other proteins are not functional in the cytoplasm or in the nucleus but are required to be present in the membrane of the cell thereby requiring the presence of leader and lipophilic sequences.

The directing of target molecules as an approach to genetic therapy has been studied by attempts at localization for the express purpose of putting an active agent such as antisense RNA in proximity to the target in a particular cellular locale For example, some workers have designed nucleic acid constructs to express anti-sense RNA that would be retained in the nucleus

in order to block newly transcribed target RNA from functioning (Izant and Sardelli, 1988, Current Communications in Molecular Biology, D. Melton, ed., Cold Spring Harbor Laboratory ; Cotten and Birnstiel, 1989, EMBO Journal 8, 3861, incorporated by reference). The opposite effect has also been achieved by designing the transcript to include a signal for enhancing transport into the cytoplasm in order to block the translation of RNA that may be present there (Liszeiwicz et al. 1993, incorporated by reference).

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SUMMARY OF THE INVENTION

The present invention overcomes the above-described limitations in the prior art by providing compositions which retain their biological function within cells or biological systems containing such cells upon chemical modification which may add further useful biological functions in addition to those which are retained.

The present invention relates to nucleic acid constructs capable of biological function and processing within a cell. These constructs may contain chemically modified biological or synthetic compounds. These constructs retain their biological function within a cell, but may also be able to exhibit additional properties by virtue of the chemical modification. The constructs combine chemical modifications and biological functions integrated within the construct.

The invention relates to novel constructs that have either incorporated unique biological elements or have incorporated chemical entities that introduce new properties to the construct, or both.

Unique biological elements are either synthetic, non-native heterologous or artificial elements in the construct that when in the cell provide novel capabilities (non-native) or novel products (artificial). Novel capabilities are

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provided by but are not limited to the introduction of such elements as heterologous processing elements that allow the construct to function in compatible cells, signaling elements for localization within the cell and multi-independent production cassettes.

Chemical modifications provide added characteristics to the constructs without interfering substantially with its biological function. Such added characteristics can be, but are not limited to nuclease resistance, the capability of targeting specific cells or specific receptors on cells, the capability of localization to specific sites within a cell, or the ability to enhance the interaction between the construct (or virus or vector) and the target cell in a general manner or too prevent or interfere with such interaction when desired.

The invention combines biological elements and chemical modification either to create a construct that defines its function, its location within a cell and its fate, or to modulate the interaction of the virus, vector or construct and cell prior to the entry of the virus, vector or construct into the cell.

Furthermore, the present invention relates to methods and constructs that provide for general interactions between target cells and a nucleic acid entity and compositions of multimeric complexes useful *in vivo* and *in vitro*.

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Among the compositions provided by this invention is a construct which when present in a cell produces a product. The construct comprises at least one modified nucleotide, a nucleotide analog or a non-nucleic acid entity, or a combination of any of the foregoing. Another composition is a construct bound non-ionically to an entity comprising a chemical modification or a ligand. When present in a cell, such a construct produces a product. Another composition provided by this invention is a construct bound non-ionically to an entity comprising a chemical modification or a ligand. When present in a cell, such a construct also produces a product.

This invention provides a composition comprising (a) a non-natural entity which comprises at least one domain to a nucleic acid component; and at least one domain to a cell of interest; and optionally, (b) the nucleic acid component, and optionally, (c) the cell of interest, or both (b) and (c). In this composition, the domain or domains to the nucleic acid component are different from the domain or domains to the cell. A kit is provided for introducing a nucleic acid component into a cell of interest. This kit comprises in packaged containers or combination one element and three optional elements. The first element is a non-natural entity which comprises at least one domain to the nucleic acid component, and a domain to the cell of interest. Optional elements include the nucleic acid component, the cell of interest, and buffers and instructions.

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Another composition provided by this invention comprises an entity which comprises at least one domain to a cell of interest, such domain or domains being attached to a nucleic acid component which is in non-double stranded form. A kit is also provided for introducing a nucleic acid component into a cell of interest.

The kit comprises in packaged containers or combination an entity which comprises a domain to the cell of interest, the domain being attached to a nucleic acid component which is in non-double stranded form. Optionally provided are buffers and instructions.

Also provided is a composition comprising an entity which comprises a domain to a nucleic acid component, the domain being attached to a cell of interest. A kit is provided for introducing a nucleic acid component into a cell of interest. In packaged containers or combination, the kit comprises an entity which comprises a domain to the nucleic acid component, the domain being attached to the cell of interest. Buffers and instructions may also be optionally included.

Further provided is a multimeric complex composition comprising more than one monomeric unit attached by means of one or more interactions. Thus, the monomeric units may be attached to each other through polymeric interactions, or to a binding matrix through polymeric interactions, or a combination of both kinds of interactions.

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Also provided is a multimeric composition comprising more than one component attached to a charged polymer. In this composition, the charged polymer is selected from a polycationic polymer, a polyionic polymer, a polynucleotide, a modified polynucleotide and a polynucleotide analog, or any combination of the foregoing elements.

The present invention provides a nucleic acid construct which when introduced into a cell codes for and expresses a non-native polymerase. The non-native polymerase is capable of producing more than one copy of a nucleic acid sequence from the construct. Also provided is a nucleic acid construct which when introduced into a cell produces a nucleic acid product comprising a non-native processing element. When contained in a compatible cell, the processing element is substantially removed during processing.

This invention also provides a process for selectively expressing a nucleic acid product in a cell, the product requires processing for functioning. The process comprises first, providing a nucleic acid construct which when introduced into a cell produces a nucleic acid product comprising a non-native processing element, which is substantially removed during processing when contained in a compatible cell, and second, introducing the construct into the cell.

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Another composition comprises a primary nucleic acid component which upon introduction into a cell produces a secondary nucleic acid component which is capable of producing a nucleic acid product, or a tertiary nucleic acid component, or both. Neither the secondary nucleic acid component, the tertiary nucleic acid component, nor the nucleic acid product are capable of producing the primary nucleic acid component.

Also provided herein is a process for localizing a nucleic acid product in a eukaryotic cell. This localizing process comprises first, providing a composition of matter comprising a nucleic acid component which when present in a cell produces a non-natural nucleic acid product. The non-natural nucleic acid product comprises a portion of a localizing entity, and a nucleic acid sequence of interest. In the second step of the process, the composition is introduced into a eukaryotic cell or into a biological system containing a eukaryotic cell.

Additionally provided by this invention is a nucleic acid component which upon introduction into a cell is capable of producing more than one specific nucleic acid sequence. Each such specific sequence so produced is substantially nonhomologous with each other and are either complementary with a specific portion of a single-stranded nucleic acid of interest in a cell or capable of binding to a specific protein of interest in a cell.

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Also contemplated by the present invention is a process for dislocating a cellular component in a cell. Here, the process comprises, comprises first, providing a nucleic acid construct which when introduced into a cell produces a non-natural product, the product itself comprising two components: a binding component capable of binding to a cellular component, and a localization component capable of dislocating the cellular component when it is bound to the non-natural product. In the second step of the process, the nucleic acid construct is introduced into a cell of interest or a biological system containing the cell or cells of interest.

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BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 depicts the localized attachment of ligands and other moieties to a nucleic acid construct by incorporation into a nucleic acid primer.

FIGURE 2 depicts the dispersed attachment of ligands to a nucleic acid construct by extension from a modified nucleic acid primer.

FIGURE 3 illustrates the dispersed attachment of ligands to a nucleic acid construct by synthesis of a complementary RNA strand that utilizes modified ribonucleotide precursors.

FIGURE 4 illustrates the localized attachment with a nucleic acid construct by hybridization of a gapped circle with a modified nucleic acid moiety that also contains useful moieties incorporated into a 3' tail.

FIGURE 5 illustrates the preparation of a gapped circle such as shown in FIGURE 4.

FIGURE 6 illustrates the localized attachment with a nucleic acid construct by hybridization of a gapped circle with a modified nucleic acid moiety with an

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unmodified 3' tail to which has been hybridized a nucleic acid with useful ligands incorporated thereinto.

FIGURES 7 AND 8 show the process for introducing a segment of RNA into a cell by means of a modified primer whereby the RNA will be transformed *in vivo* into a double-stranded DNA segment.

FIGURES 9 AND 10 show the process for introducing a segment of RNA into a cell by means of modified primers whereby the RNA will be transformed *in vivo* into double-stranded DNA segments.

FIGURE 11 illustrates a process for introducing a segment of single stranded DNA having modified nucleotides as part of its sequence.

FIGURE 12 illustrates the fate of the modified single-stranded DNA from Figure 11 after it has been introduced into a cell.

FIGURE 13 illustrates a process for introducing a segment of double stranded DNA having modified nucleotides as part of the sequence on each strand.

FIGURE 14 illustrates a divalent antibody binder with one portion having an affinity for binding a retroviral particle, and the other portion having an affinity for binding the CD34 antigen.

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FIGURE 15 shows the covalent attachment of DNA to each portion of an $F(ab')_2$ antibody fragment with an affinity for the CD34 antigen.

FIGURE 16(A) depicts the covalent attachment of DNA to an adenovirus binding portion of a divalent antibody in order to promote the binding of an AAV vector DNA molecule to a CD34 receptor.

FIGURE 16(B) is the same depiction as in FIGURE 16(A) except that $F(ab')$ fragments are used instead of complete antibody proteins.

FIGURE 17 illustrates a monovalent antibody to an adenovirus spike protein with one portion being modified by covalent attachment of DNA that can bind an adenovirus associated virus (AAV) vector DNA molecule through hybridization and the other portion being modified by the covalent attachment of an oligolysine modified by the attachment of lactyl groups.

FIGURE 18 shows a monovalent antibody to an adenovirus spike protein in which each portion of the antibody has been modified by the covalent attachment of lactosylated DNA molecules which are bound to an AAV vector DNA by means of hybridization.

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FIGURE 22 depicts a process for multimerization of insulin molecules by hybridization of nucleic acid homopolymers.

FIGURE 23 depicts a process for multimerization of insulin molecules by hybridization of nucleic acid heteropolymers with a binding matrix.

FIGURE 24 shows the introduction of an SV40 intron sequence that reconstitutes appropriate signals for in vivo splicing and production of a normal mRNA transcript for T7 RNA polymerase.

FIGURE 25 shows the process of the intron introduction and subsequent construction of a T7 expression vector.

FIGURE 26 shows the oligomers and their products used for the synthesis of the SV40 intron containing T7 RNA polymerase coding sequence.

FIGURE 27 depicts the process for the introduction of nucleotide sequences for the nuclear localization signal.

FIGURE 28 is a comparison of the 5' ends of the nucleotide sequence for the normal T7 RNA polymerase and a T7 RNA polymerase with sequences inserted for a nuclear localization signal.

FIGURE 29 shows the process for the assembly of PCR generated fragments by cloning methods to assemble a clone that directs the synthesis of an intron containing T7 RNA polymerase transcript.

FIGURE 30 shows the sequences for HIV antisense sequences and the process for their cloning into T7 directed transcription units.

FIGURE 31 shows the cloning steps for the combination of T7 directed antisense into a clone that contains the intron containing T7 RNA polymerase.

FIGURE 32 shows the DNA sequences and subsequent cloning steps for making a protein expression vector.

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FIGURE 33 shows a process for a combination of the polylinker sequence from FIGURE 32 and a T7 promoter and a T7 terminator for making a T7 directed protein expression vector.

FIGURES 34 AND 35 depicts the design of a primary nucleic acid construct that will function as a production center to generate single stranded antisense DNA.

FIGURE 36 depicts the design of a primary nucleic acid construct that will generate a secondary nucleic acid construct capable of directing transcription.

FIGURES 37 AND 38 depict the design of a primary nucleic acid construct that will generate a double hairpin production center (secondary nucleic acid construct).

FIGURE 39 depicts the design of a primary nucleic acid construct that will generate a production center (secondary nucleic acid construct) capable of inducible suicide.

FIGURE 40 depicts the design of a primary nucleic acid construct that will use tRNA primers in vivo to make secondary nucleic acid constructs capable of transcription.

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FIGURE 41 depicts the process of excision of normal sequences from a U1 transcript region and replacement with novel sequences.

FIGURE 42 shows the oligomer sequences for making HIV antisense sequences and the insertion of these oligomers as replacement for a portion of the U1 transcript sequence in a clone containing a U1 operon.

FIGURE 43 is a computer generated secondary structure prediction for U1 transcripts with HIV antisense sequence substitutions.

FIGURE 44 depicts the cloning process for making of a clone that contains multiple HIV antisense containing U1 operons.

FIGURE 45 depicts the cloning steps for constructing a clone that contains multiple independent HIV antisense containing T7 directed transcripts.

FIGURE 46 shows the final structures of the multiple operon constructs described in FIGURES 44 and 45.

FIGURE 47 depicts the cloning steps for insertion of multiple T7 antisense operons into a vector coding for the T7 intron containing RNA polymerase.

FIGURE 48 represents flow cytometry data measuring binding of anti-CD4 + antibody to HIV resistant U937 cells.

FIGURE 49 shows PCR amplification of the *gag* region indicating the absence of HIV in viral resistant cell line (2.10.16) after challenge.

FIGURE 50 depicts a model system for testing the potential inhibition of HIV antisense sequences by using beta-galactosidase activity as an indicator.

FIGURE 51 is a table of data demonstrating the effect of the HIV antisense sequence upon beta-galactosidase activity by enzyme assays as well as *in situ* assays.

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DETAILED DESCRIPTION OF THE INVENTION

Definitions

Some definitions for the terminology used in the art and/or in the present invention might be in order.

Primary Nucleic Acid Construct. A composition consisting of nucleic acid which in a cell propagates Production Centers.

Production Center. A nucleic acid molecule derived from a Primary Nucleic Acid Construct which in a cell is able to propagate other Production Centers or to produce single stranded nucleic acid.

Propagation. The generation or formation of a Production Center from a Primary Nucleic Acid Construct or the generation or formation of a Production Center from another Production Center.

Production. The generation of a single stranded nucleic molecules from a Production Center.

Inherent Cellular Systems. Cellular processes and components present in cells which can be utilized for the Production and Propagation as well as the function of single stranded Nucleic Acid Products. Such processes and

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The present invention is a defined chemically modified nucleic acid construct (CHENAC) which, upon introduction into a cell, is capable of biological function, *i.e.*, production of a nucleic acid, production of a protein in a cell or interaction with a nucleic acid or protein in a cell. The said chemical modification directly or indirectly renders the construct capable of one or more of the following properties: a) binding to a target cell b) nuclease resistance c) providing a mechanism for introduction of the nucleic acid into cells d) providing nuclease resistance within the cytoplasm e) facilitating transfer of the nucleic acid from the cytoplasm to the nucleus f) providing a longer lifetime within the cell g) providing a signal for integration into cellular DNA. In the present invention, one or more of the above properties is capable of being provided without substantially interfering with the biological function of said nucleic acid. The present invention uses chemical modification of nucleic acid to attach directly or indirectly one or more ligands or chemical modifications or other moieties to a nucleic acid construct. A construct modified by the addition of ligands or chemical modifications could further complex with other moieties, those moieties being natural or unnatural, modified or unmodified oligo- or polypeptides; polycations; natural or unnatural, modified or unmodified oligo- or

polysaccharides; multimolecular complexes; inactivated viruses; and any chemical binding, attachment or conjugation capable of complexing with the ligand or chemical moiety. The Modified Nucleic Acid Constructs of the present invention provide for the delivery of nucleic acid to eucaryotic cells including the cells of plants, humans and other mammals and to procaryotic cells.

The present invention provides the capability to localize chemical modifications to regions of the CHENAC. This permits construction of compositions in which the segment of the CHENAC responsible for the biological function can be segregated from modified region(s) responsible for the properties listed above in cases where the addition of ligands or chemical modifications could be disruptive to biological function. In cases where ligands or chemical modifications can interfere with biological activity, chemically modified segments of the CHENAC could be segregated from the construct subsequent to introduction into the cell by displacement or loss of modified segments.

In one aspect, this invention provides a construct which when present in a cell produces a product, the construct comprising at least one modified nucleotide, a nucleotide analog and a non-nucleic acid entity, or a combination of the foregoing. The modified nucleotide may be chemically modified as described further below. When present in the construct, at least

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one of the nucleotide analog or analogs may also be modified either on the backbone or the side chain or on both positions. With respect to the non-nucleic acid entity this element may also be attached to a single strand or both strands of the construct when the latter is double stranded.

The non-nucleic acid entity or entities may take any number of diverse forms. These include natural polymers, synthetic polymers, natural ligands and synthetic ligands, as well as combinations of any and all of the foregoing.

When the non-nucleic acid entity or entities take the form of a natural polymer, suitable members may be modified or unmodified. Natural polymers can be selected from a polypeptide, a protein, a polysaccharide, a fatty acid, and a fatty acid ester as well as any and all combinations of the foregoing.

When the present invention contemplates the use of a synthetic polymer for the non-nucleic acid entity or entities, homopolymers and heteropolymers may be employed. Such homopolymers and heteropolymers are in many ways preferred when they carry a net negative charge or a net positive charge.

It is significant that the above-described construct of the present invention can be designed to exhibit a further and additional biological activity which is usefully imparted by incorporating at least one or more modified nucleotides, nucleotide analogs, nucleic acid entities, ligands or a combination of any or

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all of these. Such biological activity may itself take a number of forms, including nuclease resistance, cell recognition, cell binding, and cellular (cytoplasmic) or nuclear localization.

The nucleic acid of the CHENAC can be DNA, RNA, a combination of RNA and DNA, e.g., a DNA-RNA hybrid or a chimeric nucleic acid, such as a DNA-RNA chimera. The nucleic acid components of the CHENAC can be single-stranded or double-stranded. The nucleic acid component can be all or in part a modified nucleic acid or a nucleic acid analogue. Modified nucleic acids are polymers capable of binding to complementary regions of nucleic acids and which contain chemical modifications of the sugar, base or phosphate moieties.

Nucleic acid analogues are polymers capable of binding to a complementary nucleic acid and in which these polymer backbones are other than ribo- and deoxyribose sugars and phosphate groups or in which side chain groups are other than natural or modified bases. Examples of nucleic acid analogue polymers include peptide nucleic acids or which have side chains containing such non-discriminatory base analogues, or universal bases, as 1-(2'-deoxy- β -D-ribofuranosyl)-3-nitropyrrole (Nichols et al. 1994 *Nature* **369**:492,) or 2'-deoxynebularine and 2'-deoxyxanthosine (Eritja et al. 1986 *Nucleic Acids Research* **14**:8135), both publications being incorporated herein by reference..

Modified nucleic acids, nucleic acid analogues and other polymers with a net negative charge and/or a functional amino group(s) may facilitate the practice of this invention, since these properties provide for solubility, specificity, enzyme function and binding. It may be preferred that some of the functional sequences of nucleic acid may be natural or modified nucleic acid sequences such as promoter sequences, terminator sequences or priming binding sequences.

The nucleic acid component of the CHENAC can be single stranded, double stranded, partially double stranded or even triple stranded. Further, such component can be circular or linear or branched, and may take the form of any DNA or RNA. It can contain both double stranded and single stranded regions and it can contain an non-complementary region, e.g, a tail. Such a tail region could further be bound to complementary nucleic acid. For example, single stranded nucleic acid can be present as one or more regions of single stranded DNA as a gap between otherwise continuous double stranded structure (see Figure 3, Gap 2). Alternatively, linear single stranded nucleic acid can be present as tails, or linear single stranded nucleic acids in which one end is bound to the CHENAC and the other end is free (See Figure 4 and 6a). Gaps and tails can be single stranded RNA or DNA or a variety of other polymers both natural and synthetic, including modified nucleic acids, nucleic acid analogues, polysaccharides, proteins and other natural and

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Ligands or chemical modifications can be attached to the nucleic acid, modified nucleic acid or nucleic acid analogue by modification of the sugar, base and phosphate moieties of the constituent nucleotides (Engelhardt et al., US Patent No. 5,260,433, fully incorporated herein by reference) or to a non nucleic acid segment of the CHENAC such as polysaccharide, polypeptide and other polymers both natural and synthetic. Modifications of sugar and phosphate moieties can be preferred sites for terminal binding of ligands or chemical modifications and other moieties. Modifications of the base moieties can be utilized for both internal or terminal binding of ligands or chemical modifications and other moieties. Modifications which are non-disruptive for biological function such as specific modifications at the 5 positions of pyrimidines (Ward et al. U.S. Patent No. 4,711,955, and related divisionals) and the 8 and 7 positions of purines (Engelhardt et al., U.S. Patent No. 5,241,060 and related divisionals; Stavrianopoulos, U.S. Patent No. 4,707,440 and related divisionals) may be preferred. The contents of

each of the aforementioned U.S. patents and their related divisionals are incorporated herein by reference.

Chemical modification can be limited to a specific segment of the construct such as a tail or a gap, or dispersed throughout the CHENAC. Thus, the construct may contain at least one terminus, such a terminus comprising, for example, a polynucleotide tail. Such a modified nucleic acid, subsequently introduced into a cell, could be displaced and /or replaced.

In a further embodiment the present invention provides the construct, described above, further comprising at least one ligand attached covalently or noncovalently to one or more of the modified nucleotide analogs, nonnucleic acid entities (or combinations of the foregoing). Such ligands and chemical modifications can be added directly to the CHENAC through covalent and non-covalent interactions. Covalent additions can be made by chemical methods (Engelhardt et al.) and enzymatic incorporation. Non-covalent additions can be made through nucleic acid-nucleic acid interaction, antigen-antibody interaction, hydrophobic interaction and other interactions based on nucleic acid sequence, nucleic acid structure, protein structure. Indirect additions to the CHENAC can be made by these same methods and interactions. When included in the present invention, such ligand or ligands are attached to any portion or any form of the present construct. Thus the ligand or ligands can be attached to a single stranded segment, a double

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stranded segment, a single stranded construct tail, a sequence complementary to a construct tail or to any combinations of these portions or forms.

Ligands or chemical modifications, being any chemical entity, natural or synthetic, which can be utilized in this invention include macromolecules greater than 20,000 m.w. as well as small molecules less than 20,000 m.w. The ligand or ligands can include both macromolecules and small molecules. Macromolecules which can be utilized include a variety of natural and synthetic polymers including peptides and proteins, nucleic acids, polysaccharides, lipids, synthetic polymers including polyanions, polycations, and mixed polymers. Small molecules include oligopeptides, oligonucleotides, monosaccharides, oligosaccharides and synthetic polymers including polyanions, polycations, lipids and mixed polymers. Small molecules include mononucleotides, oligonucleotides, oligopeptides, oligosaccharides, monosaccharides, lipids, sugars, and other natural and synthetic entities.

Ligands and chemical modifications provide useful properties for nucleic acid transfer such as 1) cell targeting entities, 2) entities which facilitate cellular uptake, 3) entities specifying intracellular localization, 4) entities which facilitate incorporation into cellular nucleic acid and 5) entities which impart nuclease resistance.

1) Cell targeting entities which can be utilized include:

a) antibodies to cellular surface components and epitopes

b) viruses, virus components or fragments of virus components which have affinity for cellular surface components. These include such proteins as the gp120 protein of HIV which binds to the CD4 receptor of T4 lymphocytes (Lever 1995 British Medical Bulletin 51;149, incorporated herein by reference).

c) ligands which have affinity for cell surfaces. These include hormones, lectins, proteins, oligosaccharides and polysaccharides. Asialoorosomucoid, for example, binds to the cellular asialoglycoprotein receptor (Wu et al. 1989 J Biol Chem 269;16985, incorporated herein by reference) and transferrin binds to transferrin cellular receptors (Wagner et al. 1992 89; 6099, also incorporated herein by reference).

d) polycations such as polylysine that bind nonspecifically to cell surfaces (Wu and Wu)

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e) Matrix proteins such as fibronectin that bind to hematopoietic cells and other cells (Ruoslahti et al. 1981 J. Biol. Chem. **256**;7277, incorporated by reference),

f) lectins which bind to cell surface components.

Entities which facilitate cellular uptake include inactivated viruses such as adenovirus (Cristiano et al. 1993 Proc Natl Acad Sci USA **90**;2122; Curiel et al. 1991 Proc Natl Acad Sci USA **88**;8850, all of which are incorporated by reference); virus components such as the hemagglutinating protein of influenza virus and a peptide fragment from it, the hemagglutinin HA-2 N-terminal fusogenic peptide (Wagner et al. 1992 Proc Natl Acad Sci USA **89**;7934, also incorporated herein by reference).

Entities which specify cellular location include:

- a) nuclear proteins such as histones
- b) nucleic acid species such as the snRNAs U1 and U2 which associate with cytoplasmic proteins and localize in the nucleus (Zieve and Sautereau 1990 Biochemistry and Molecular Biology **25**;1, incorporated by reference)

4) entities which facilitate incorporation into cellular nucleic acid include:

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a) proteins which function in integration of nucleic acid into DNA. These include integrase site specific recombinases (Argos et al. 1986 EMBO Journ 5; 433, incorporated by reference); and

b) homologous nucleotide sequences to cellular DNA to promote site specific integration.

5) Entities which impart nuclease resistance modifications of constituent nucleotides including addition of halogen atoms groups to the 2' position of deoxynucleotide sugars. (Braket et al., U.S. Patent Application Serial No. 07/446,235, filed on December 4, 1989, incorporated by reference).

Ligands or chemical modifications can be introduced into CHENACs either

a) directly by conjugation, b) by enzymatic incorporation of modified nucleoside triphosphates c) by reaction with reactive groups present in constituent nucleotides and d) by incorporation of modified segments. These processes include both chemical and enzymatic methods. Enzymatic methods include primer extension, RNA and DNA ligation, random priming (Kessler et al. 1990 Advances in Mutagenic Research, Vol. 1, Springer Verlag, pp 105 -152), nick translation (Rigby et al., 1977, J. Mol. Biol. 113, 237), polymerase chain reaction (Saiki et al., 1985 Science 239, 487), RNA labeling methods utilizing T7, T3 and SP6 polymerases, (Melton et al. 1984 Nucleic Acids Research 12, 7035; Morris et al. 1986 Gene 41,193), terminal

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Variable	Mean		SD		t		p	
	Control	Case	Control	Case	Control	Case	Control	Case
Age	24.5	24.5	2.5	2.5	0.0	0.0	1.000	1.000
Sex	10	10	1	1	0.0	0.0	1.000	1.000
Height	170.0	170.0	5.0	5.0	0.0	0.0	1.000	1.000
Weight	65.0	65.0	10.0	10.0	0.0	0.0	1.000	1.000
Heart rate	70.0	70.0	10.0	10.0	0.0	0.0	1.000	1.000
Blood pressure	120.0	120.0	10.0	10.0	0.0	0.0	1.000	1.000
Respiratory rate	12.0	12.0	2.0	2.0	0.0	0.0	1.000	1.000
SpO ₂	98.0	98.0	1.0	1.0	0.0	0.0	1.000	1.000
Temperature	36.5	36.5	0.2	0.2	0.0	0.0	1.000	1.000
Heart rate variability	50.0	50.0	10.0	10.0	0.0	0.0	1.000	1.000
Respiratory rate variability	1.0	1.0	0.2	0.2	0.0	0.0	1.000	1.000
SpO ₂ variability	0.5	0.5	0.1	0.1	0.0	0.0	1.000	1.000
Temperature variability	0.1	0.1	0.02	0.02	0.0	0.0	1.000	1.000
Heart rate variability (HRV)	50.0	50.0	10.0	10.0	0.0	0.0	1.000	1.000
Respiratory rate variability (RRV)	1.0	1.0	0.2	0.2	0.0	0.0	1.000	1.000
SpO ₂ variability (SpO ₂ V)	0.5	0.5	0.1	0.1	0.0	0.0	1.000	1.000
Temperature variability (TempV)	0.1	0.1	0.02	0.02	0.0	0.0	1.000	1.000

CHENACs can be prepared by the incorporation of nucleic acid segments modified by ligands or chemical modifications. Constructs can also be prepared by the incorporation of unmodified nucleic acid segments together with other segments. Segments incorporated into constructs can be single stranded or double stranded or composed of both single and double stranded regions. Such segments can be composed of DNA, RNA, a combination of DNA and RNA, or chimeric nucleic acids. All or part of a segment can be composed of modified nucleic acid or nucleic acid analogue. All or part of a segment can contain natural or synthetic polymers. A segment can be

prepared by any of the chemical methods and enzymatic methods listed above.

The present invention provides for choice of localization of ligands or chemical modifications. In order that such ligands or chemical modifications do not interfere with biological activity segments with biological activity can be isolated from modified segments in the CHENAC. Also, modifications can be confined to a region of a segment. For example, a specific primer labeled with Ligands or chemical modifications of choice can be hybridized to a defined region of the construct, and polymerization can be done in the presence unmodified nucleotides in order to confine the ligands or chemical modifications to a defined area of the primer. Alternatively, an unmodified primer can be used to synthesize in the presence of modified nucleotides to confine the ligands or chemical modifications to the non-primer region of the strand. Alternatively, by using a primer containing ligands or chemical modifications, labeling can be done throughout the strand or through complementarity to a tail.

Regions of biological activity in constructs can specify coding for RNA (such as antisense RNA or ribozymes as described in this patent, Example 26) or for RNA which is translated into protein or for DNA. Regions of biological activity in CHENACs can contain sequences for hybridization with intracellular nucleic acid sequences, integration into cellular DNA, termination

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sequences, primer sites, promoter sites and processing signals and sequences.

In one preferred embodiment the construct of the present invention carries a net positive charge or a net negative charge. Further, the construct can be neutral or even hydrophobic. It should not be overlooked that the construct may comprise unmodified nucleotides and at least one other member or element selected from one or more nucleotide analogs and non-nucleic acid entities, or both.

Another significant embodiment of the present invention is a construct which when present in a cell produces a product, the construct being bound non-ionically to an entity comprising either a chemical modification or a ligand addition, or both. As in the case of the other above-described construct, this construct may also comprise at least one terminus, such terminus comprising a polynucleotide tail. The polynucleotide tail is hybridizable or hybridized to a complementary polynucleotide sequence. An antibody to a double stranded nucleic acid can be directed and thus bound to such hybridized polynucleotide tail sequences. The antibody can comprise a polyclonal antibody or a monoclonal antibody.

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Nucleic Acid Component: a compound or composition in a cell capable of producing a product. The composition comprises a nucleic acid sequence desired to be delivered to a cell including polynucleotide, modified nucleic acid and nucleic acid analogues which can be single stranded or double stranded RNA or DNA, RNA/DNA hybrid molecules and chimeric nucleic acids; nucleic acid construct and chemically modified nucleic acid constructs (See Examples 1 through 13); viruses including animal viruses such as adenovirus, adeno associated virus, retrovirus and plant viruses and bacteriophages; plasmids including the Ti plasmid; or plasmid derivatives that have encapsidated into viral particles by virtue of packaging signals. Nucleic Acid Components can be produced *in vivo* or assembled *in vitro* or produced chemically or produced by the techniques of recombinant DNA. The product produced from the Nucleic Acid Component in the cell could be a polynucleotide including mRNA, antisense RNA or DNA, ribozymes or it could be a protein or a protein product.

Domain: A Domain is an entity that has a segment that binds non covalently either to a cell or to a Nucleic Acid Component.

Binder: A Binder is a carrier or matrix that includes at least one Domain.

The present invention overcomes the limitations of prior art by providing a composition and method for universal and efficient nucleic acid transfer. The nucleic acid, whether in a virus vector, in a nucleic acid construct or as polynucleotide, can be introduced into a wide variety of cell types.

Furthermore, the use of virus vectors in this invention is not limited to a specific or a unique viruses but a wide variety of virus vectors can be used. This invention is universal in two respects: 1) any Nucleic Acid Component can be applied either *in vivo* or *in vitro* and 2) any target cell can be used.

In the practice of this invention it is possible to:

- 1) bring into close proximity the Nucleic Acid Component and the target cell; and
- 2) provide specificity between the Nucleic Acid Component and the target cell.
- 3) enhance nucleic acid transfer to the cell by providing Competence Factors which enhance nucleic acid transfer through enhancing cell growth, cellular uptake of nucleic acid, cellular localization of nucleic acid and integration of nucleic into cellular DNA.

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The present invention provides materials and methods for the delivery of nucleic acids to cells. The specificity and/or proximity are provided through an intermediate, a Binder which consists of at least one Domain. If the Binder has at least one Domain to the target cell, then the Binder is attached to a Nucleic Acid Component. If the Binder has at least one Domain to a Nucleic Acid Component, then the Binder is attached to a target cell. If the Binder has at least one Domain to both the Nucleic Acid Component and the target cell the Domain to the cell is different from the Domain to the Nucleic Acid Component.

One of the significant embodiments of the present invention is a composition comprising a non-natural entity which in turn comprises at least one domain to a nucleic acid component; and at least one domain to a cell of interest. The domain or domains to the nucleic acid component are different from the domain or domains to said cell. Optional elements may be added to this composition or non natural entity including the nucleic acid component, the cells of interest, or both such nucleic acid component and such cells.

The entity can, of course, comprise a binder. Further, the binder and the domain in the non natural entity can be the same or they can be different.

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A Binder is a support or matrix that is composed of at least one Domain. A Binder can be natural or synthetic, such as a polymer, support, matrix or carrier (or combination of these). The binder comprises at least one Domain to a Nucleic Acid Component or to a cell of interest or to both. As such, the Binder can be a monofunctional or bifunctional entity. In the case of a monofunctional Binder, only one Domain is present, either to the Nucleic Acid Component or the cell of interest. In the case of a bifunctional binder, at least two domains are present, one to the Nucleic Acid Component and the other to the cell of interest. Where two domains are present in the binder, i.e., a bifunctional binder, the domain to the Nucleic Acid Component is different from the Domain to the cell of interest. In some cases Domains and Binders can be the same entity, such as an antibody that has a segment (an Fab region) that binds to an epitope and has an Fc segment that can function as a support for attachment.

A Domain is an entity that has a segment that binds either to a cell or to a Nucleic Acid Component. Domains can be natural or synthetic polymers including oligopeptides, polypeptides, oligosaccharides, polysaccharides, oligonucleotides and polynucleotides. These include monoclonal antibodies, polyclonal antibodies, polycations such as polyamines, ligands to cell surface proteins, extracellular matrix proteins and ligands and their binding partners. These can be produced *in vivo* or assembled *in vitro* or produced chemically or produced by recombinant DNA techniques.

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Domains provide binding to cells or to NA Entities through specific or non-specific binding through a variety of interactions including nucleic acid-nucleic acid interaction, antigen-antibody interaction, receptor-ligand interaction, hydrophobic interaction, polyionic interaction and other interactions based on nucleic acid specificity, nucleic acid sequence and proteins capable of specifically binding to such sequences or secondary structures or combinations thereof. Interactions between ligand binding pairs and between complementary nucleic acid sequences may be preferred for the application of this invention. These include a nucleotide sequence recognized by a complementary sequence, an antigen by an antibody, a lectin recognized by its cognate sugar, a hormone recognized by its receptor, an inhibitor recognized by an enzyme, a cofactor recognized by its cofactor enzyme binding site, a binding ligand recognized by its substrate and combinations of the foregoing.

Antibodies provide useful Domains. Monoclonal and polyclonal antibodies and fragments of these can be used. Antibodies can be obtained from sera, from hybridomas and by recombinant DNA methods. Bispecific antibodies which have the capability to bind to two different epitopes can also be useful. These can be hybrid hybridomas (Staerz and Bevan 1986 Proc Natl Acad Sci USA 83;1453), heteroantibodies produced by chemical conjugation of antibodies, or fragments of antibodies, of different specificities (Fanger et

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al. 1992 Critical Rev Immunol. 12;101), bispecific single chain antibodies (Gruber et al. 1994 Journ Immunol 152;5368) produced by genetic engineering and diabodies (Holliger et al. 1993 Proc Natl Acad Sci USA 90;6444) produced by genetic engineering. All of the foregoing publications are incorporated herein by reference.

Useful Domains with non-specific cell binding properties include molecules with polyionic properties such as polycations including polylysine, protamine, histones or segments or fragments thereof.

Useful Domains with specific cell binding properties include:

1) those with binding affinity for a natural cell component, epitope or ligand.

Such cell binding domains include ligands specific to cell receptors such as hormones, mono- and oligosaccharides, viral proteins which recognize cell receptor sites, extracellular matrix proteins such as fibronectin and fragments thereof, antibodies to cell proteins and fragments thereof.

2) those with binding affinity for a non-naturally introduced ligand where a) the ligand is attached to a cell by chemical means such as by reaction with a tyrosine or amino group of a cellular surface protein or b) the ligand is indirectly attached to a cell non-specifically.

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Useful Domains with non-specific Nucleic Acid Component binding properties include those which bind non-covalently and not through a ligand/receptor system. Examples are polycations such as polylysine and histones that bind to nucleic acid.

Useful Domains with specific Nucleic Acid Component binding properties include:

- 1) those with binding affinity for a natural component of a Nucleic Acid Component, epitope or ligand. These include:
 - a) antibodies to nucleic acid including antibodies to double stranded and single stranded DNA, to double and single stranded RNA or to RNA/DNA hybrids; proteins with nucleic acid binding properties such as the Cro protein of bacteriophage lambda which binds to a sequence of 17 base pairs (Anderson et al. 1981 Nature 290;754, incorporated by reference).
 - b) antibodies to an epitope or receptors for a ligand of a Nucleic Acid Component. These include antibodies to viral proteins, cellular receptors and virus binding proteins, such as the CD4 protein of lymphocytes.
- 2) artificial specific binding systems (Domains) can be formed by chemically introducing a ligand to the Nucleic Acid Component where said ligand has a corresponding receptor. Such specific ligands or

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epitopes can be artificially introduced by chemical modification of a tyrosine or amino group of, for example, a vector virus protein.

Binders possessing two Domains can exist naturally or they can be prepared synthetically or artificially. For example, a Binder which possesses one Domain with cell binding capabilities can be associated with a Domain with Nucleic Acid Component binding capabilities to form a bifunctional Binder. This association can occur by 1) by covalent attachment 2) by specific non-covalent attachment and 3) by non-specific non-covalent attachment or 4) as a fusion peptide prepared by recombinant DNA techniques.

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In the above-described composition of this invention the nucleic acid component can take a number of different forms including a nucleic acid, a nucleic acid construct, a virus, a viral fragment, a viral vector, a viroid, a phage, a plasmid, a plasmid vector, a bacterium, and a bacterial fragment as well as combinations of these. The cell of interest can be prokaryotic or eukaryotic. As described elsewhere in this disclosure the domains can be attached noncovalently or through a binder or through combinations of these. Where noncovalent binding is used, ionic interactions and/or hydrophobic interactions are preferred. In addition the noncovalent binding can comprise a specific complex, e.g., a specific complex mediated by a ligand binding receptor. The ligand binding receptor can itself take a number of forms. Suitable but not necessarily limited to these members are a polynucleotide sequence to be recognized by its complementary sequence; an antigen to be recognized by its corresponding monoclonal or polyclonal antibody, an antibody to be recognized by its corresponding antigen; a lectin to be recognized by its corresponding sugar; a hormone to be recognized by its receptor; a receptor to be recognized by its hormone; an inhibitor to be recognized by its enzyme; an enzyme to be recognized by its inhibitor; a cofactor to be recognized by its cofactor enzyme binding site; a cofactor enzyme binding site to be recognized by its cofactor; a binding ligand to be recognized by its substrate; or a combination of the foregoing.

Another aspect of the present invention concerns the composition, described above, wherein the domain to the nucleic acid component and the domain to the cell of interest are natural, and the binder is attached to the nucleic acid component by means other than a natural binding site. Here, as in other embodiments, the binder can comprise modified fibronectin or modified polylysine or both.

Cells of interest containing or associated with the above-described compositions may be contained within a biological system, such as an organism.

Also provided are methods for introducing a nucleic acid component, as described above, into a cell. Essentially the method comprises providing any of the above-described compositions and administering these to an appropriate biological system. Administration can be carried out in vivo or ex vivo.

This invention also contemplates kits which are useful for introducing a nucleic acid component into a cell of interest. These kits comprise in packaged containers or combination a non-natural entity which comprises at least one domain to a nucleic acid component, and at least one domain to the cell of interest. Optionally included in such kits are the nucleic acid components, the cells of interest and buffers and instructions.

Another significant embodiment is a composition comprising an entity which comprises at least one domain to a cell of interest, wherein the domain or domains are attached to a nucleic acid component which is in non double

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stranded form. As elsewhere, the entity can comprise a binder, and the binder in the domain can be the same or they can be different. Among others the binder can comprise a polymer, a matrix, a support or a combination of these. The cell of interest can be prokaryotic or eukaryotic. As also described above, the nucleic acid component can take a number of forms including but not limited to a nucleic acid, nucleic acid construct, nucleic acid conjugate, a virus, a viral fragment, a viral vector, a viroid, a phage, a plasmid, a plasmid vector, a bacterium, and a bacterial fragment or combinations of these. The domain can comprise covalent bonding or noncovalent binding, or both. Preferred as noncovalent binding are ionic interactions and hydrophobic interactions (or both), and a specific complex e.g., a specific complex mediated by a ligand binding receptor. Such ligand binding receptors have been described above. The cell of interest which is part of the composition may be contained within an organism. This last described composition can likewise be usefully employed in a method for introducing a nucleic acid component into a cell. This process has also been described above.

Kits for introducing a nucleic acid component into a cell of interest can be fashioned from this composition. Such a kit comprises in packaged containers or combinations an entity which comprises a domain to a cell of interest, wherein the domain is attached to a nucleic acid component which is in non-double stranded form. Buffers and instructions may be optionally included.

This invention also provides a composition comprising an entity which comprises a domain to a nucleic acid component wherein the domain is attached to a cell of interest. As further embodiments of this just described composition are the entity, the binder, the domain, nucleic acid component, the cell of interest, the covalent bonding and noncovalent binding of the domain, the ionic and hydrophobic

interactions, the specific complex (including its mediation by a ligand binding receptor), the ligand binding receptor, as well as organisms, methods and kits for introducing nucleic acid components into cells containing the cell of interest are all as variously described above.

ATTACHMENT OF NUCLEIC ACID COMPONENTS TO MONOFUNCTIONAL BINDERS

1) Covalent Attachment of a Nucleic Acid Component to a monofunctional Binder which possesses a Domain to a cell.

Covalent attachment can occur by direct coupling between reactive groups inherent to a Domain or Binder or by the use of a bifunctional crosslinker. Also, reactive groups can be introduced into Domains and Binders in order to facilitate such covalent attachment. Attachment to proteins, for example, can occur through reactive amino groups or tyrosine residues. Attachment can be made by protein-protein conjugation. Covalent attachment can also be made to polysaccharides and to polynucleotides. Covalent attachment to a nucleic acid, modified nucleic acid or nucleic acid analogue can be made through modification of the sugar, base or phosphate moieties of the constituent nucleotides (Engelhardt et al., US Patent No. 5,260,433, incorporated by reference). Also, nucleotide analogues can be introduced

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into nucleic acid to provide reactive groups, e.g., allylamine groups (Ward et al. U.S Patent No. 4,771,955 and divisionals, also incorporated herein by reference) and proteins can be covalently attached to these as described below using N-maleimido tri(aminocaproic) acid N-hydroxysuccinimide ester as a bifunctional coupler. Modifications of sugar and phosphate moieties can be preferred sites for terminal attachment of ligands and other moieties. Modifications of the base moieties can be utilized for both internal or terminal attachment of ligands and other moieties. Modifications can include those which are non-disruptive for hybridization such as specific modifications at the 5 positions of pyrimidines (Ward et al., U.S. Patent No. 4,711,955 and related divisionals). Modifications of the 8 and 7 positions of purines (Englhardt et al. U.S. Patent No. 5,241,060 and related divisionals) and Stavrianopoulos, U.S. Patent No. 4,707,440 and related divisionals) may be preferred. In one embodiment, the chemical modification in the construct or construct components may be effected to a moiety independently selected from a base, a sugar, and a phosphate, or a combination of any or all three.

Direct covalent attachment of a Nucleic Acid Component to a

Monofunctional Binder can be illustrated by attachment of a double stranded DNA molecule (the Nucleic Acid Component) to an antibody which binds to a cell surface component (a monofunctional Binder). For example, an antibody which binds to the CD4 component of lymphocytes can be covalently attached to a double stranded DNA (a Nucleic Acid Component) which has

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been modified by the incorporation of nucleotides containing allylamine in order to provide a primary amine as a reactive group. The covalent attachment can be made as described below using N-maleimido tri(aminocaproic) acid N-hydroxysuccinimide ester as a bifunctional coupler.

Fibronectin can also be used for the covalent attachment of a Nucleic Acid Component for delivery of nucleic acid to cells. For example, fibronectin, a fibronectin fragment or fibronectin containing compounds can be attached to either a polynucleotide or to a virus vector. For example, fibronectin can be covalently attached to an allylamine group of a Nucleic Acid Component. A virus vector Nucleic Acid Component, such as adenovirus, can also be covalently bound to fibronectin by protein-protein conjugation. The covalent attachment can be made as described below using N-maleimido tri(aminocaproic) acid N-hydroxysuccinimide ester.

2) Specific non-covalent attachment of a nucleic acid component to a monofunctional binder which possesses a Domain to a cell.

Non-covalent attachment of a Nucleic Acid Component can occur through complementary nucleic acid binding. A Binder composed of an antibody to a cell surface protein can be covalently coupled to a single stranded DNA by allylamine groups incorporated into the DNA as described below using N-maleimido triaminocaproic acid N-hydroxysuccinimide ester as a bifunctional coupler. The single stranded DNA is attached through complementarity to a

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tail sequence of a Nucleic Acid Component. For example, an antibody to a CD4 cell receptor can be covalently attached to a single stranded DNA molecule which is complementary to the single stranded tail of a construct (such as the one described in Example 6) to deliver nucleic acid to CD4 + cells.

Fibronectin can be modified to provide for the non-covalent attachment of a Nucleic Acid Component. Fibronectin can be covalently attached to an antibody which has binding specificity for a virus vector such as adenovirus. Fibronectin and anti-adenovirus antibody are covalently attached by the use N-maleimido tri(aminocaproic) acid N-hydroxysuccinimide ester as a bifunctional coupler as described below.

3) Non-specific non-covalent attachment of a Nucleic Acid Component to a monofunctional Binder which possesses a Domain to a cell.

This can be achieved by the non-covalent attachment of a Domain, such as polylysine which binds to polynucleotides (Nucleic Acid Component).

Polylysine can attach to a monofunctional Binder composed of a DNA oligomer modified by the covalent addition of trilactyl lysyl lysine (Domain to a cell) as described in Example 1 of this patent. The resulting entity can deliver nucleic acid specifically to liver cells.

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ATTACHMENT OF CELLS TO MONOFUNCTIONAL BINDERS WITH DOMAINS TO A NUCLEIC ACID COMPONENT.

1) Covalent attachment of a cell to a monofunctional Binder which possesses a Domain to a Nucleic Acid Component

A Binder with a Domain for a Nucleic Acid Component can be covalently attached to a cell. For example, a monoclonal antibody to adenovirus can be covalently attached to a cell to provide adenovirus binding sites on the cell surface. Covalent attachment of the antibody can be made by the use of N-maleimido tri(aminocaproic) acid N-hydroxysuccinimide ester as a bifunctional coupler.

Synthesis of the bifunctional coupler and its use for covalent attachment of proteins is described. Tri(aminocaproic) acid is reacted with a threefold excess of 3-maleimidopropionic acid N-hydroxysuccinimide ester at a pH 7.8 for 30 minutes at room temperature. The pH is adjusted to 4.0 with acetic acid and the solution is freeze dried. The solid is triturated with ethanol to remove unreacted 3-maleimidopropionic acid active ester and traces of ethanol are removed in vacuum. The solid residue is dissolved in dimethylformamide and filtered from the insoluble salts and reacted with 1.1 equivalents of dicyclohexyl carbosuccinimide at room temperature overnight. The hydroxyurea is removed by filtration and the dimethylformamide is removed in high vacuum at 35°C. The semisolid residue is triturated with isopropanol to remove unreacted dicyclohexylcarbodiimide and N-

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At the end of the reaction the cells are reacted with 0.5 mM cysteine in phosphate buffered saline to reconstitute any blocked thiol residues on the cell surface, and the cells are washed by centrifugation in phosphate buffered saline.

This can be accomplished by the covalent attachment of biotin to cell surface proteins using an N-hydroxysuccinimide ester of biotin (Enzotin, Enzo

Biochem, Inc.). A binder composed of an antibody to adenovirus covalently attached (by the Fc portion) to avidin will bind to biotin molecules on the cell surface to provide adenovirus binding to the cell surface.

3) Non-specific non-covalent attachment of a cell to a monofunctional Binder with a Domain for a Nucleic Acid Component.

Polylysine can be covalently attached to the Fc portion of an antibody to adenovirus. The polylysine/anti-adenovirus antibody will bind to the cell surfaces to provide attachment sites for an adenovirus vector.

BINDING OF CELLS TO NUCLEIC ACID COMPONENTS THROUGH
BIFUNCTIONAL BINDER MEDIATION

Such bifunctional Binders can be formed by the attachment of two Domains either directly or through a binder or a matrix. The attachment can be covalent, non-covalent, non-specific non-covalent or specific non-covalent.

Specific attachment of cells to Nucleic Acid Components can be accomplished by the use of a bifunctional Binder. Such a Binder can be prepared by the association of a domain for a Nucleic Acid Component with a Domain for a cell. For example, an antibody to adenovirus can be covalently attached by the Fc portion to polylysine. An antibody to a cell surface protein such as CD4 can also be covalently attached to the polylysine to produce a bifunctional Binder.

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- 1) entities which enhance cell growth. These include extracellular matrix proteins such as fibronectin, which enhance the growth and the transformation efficiency of cells.

2) entities which facilitate cellular uptake. These include inactivated viruses such as adenovirus (Cristiano et al. 1993

Proc Natl Acad Sci USA 90;2122: Curiel et al. 1991 Proc Natl Acad Sci USA 88;8850, all of which are incorporated herein by reference), virus components such as the hemagglutinating protein of influenza virus and a peptide fragment from it, the hemagglutinin HA-2 N-terminal fusogenic peptide (Wagner et al. 1992 Proc Natl Acad Sci USA 89;7934, incorporated by reference).

- 3) entities which facilitate incorporation of nucleic acid into cellular nucleic acid. These include integrase site specific recombinases (Argos et al. 1986 EMBO Journal 5; 433, also incorporated by reference).
- 4) entities which function in cellular localization of nucleic acid. These include nuclear proteins such as histones and nucleic acid species such as the snRNAs U1 and U2 which associate with cytoplasmic proteins and localize in the nucleus (Zieve and Sautereau 1990 Biochemistry and Molecular Biology 25;1, incorporated by reference).

Factors unattached to a Nucleic Acid Construct, a Binder or a Domain can also facilitate nucleic acid transfer by increasing the competence of cells for nucleic acid transfer. These include factors which act to promote cell growth

and are be added to target cells during, before or after the process of gene transfer in vivo or ex vivo. These include:

- 1) growth factors such as IL-3, IL-6, GM-CSF, Epo and SCF which stimulate cell growth (Palsson et al., 1993 Biotechnology 11;368: Koller et al. 1993 Biotechnology 11;358: Koller et al. 1993 Blood 82;378, both of which are incorporated by reference) and
- 2) entities such as matrix proteins, their fragments or compounds containing these moieties, e.g., fibronectin, which form a cell binding matrix which promotes cell growth.

The present invention provides one or more of such effects in vivo or ex vivo. Such in vivo or ex vivo effects include the following:

- 1) bringing a Nucleic Acid Component and a target cell into close proximity
- 2) providing specificity for the interaction between the Nucleic Acid Component and the target cell.

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The present invention provides novel methods and compositions to form multimeric complexes in which the individual components enjoy retention of their monomeric activity while also maintaining solubility after being joined together. Such a multimeric complex consists of more than one monomeric unit, either bound to each other noncovalently through a polymeric interaction or noncovalently bound to a matrix by a polymeric interaction.

The present invention provides a multimeric complex composition comprising more than one monomeric unit attached to each other through polymeric

interactions or attached to a binding matrix through polymeric interactions or a combination of both interactions. The polymer or oligomer of the monomeric unit can be linear or branched, and it can comprise a homopolymer or a heteropolymer. The monomeric unit can comprise an analyte-specific moiety such as one which is capable of recognizing a component in a biological system, e.g., a virus, a phage, a bacterium, a cell or cellular material, a tissue, an organ or an organism, or combinations thereof.

The analyte-specific moiety can take a number of forms including its derivation or selection from a protein, a polysaccharide, a fatty acid or fatty acid ester and a polynucleotide (linear or circular or single stranded) or a combination of these. As an analyte-specific moiety such a protein can comprise an antibody (polyclonal or monoclonal), a hormone, a growth factor, a lymphokine or a cytokine, and a cellular matrix protein, or a combination of these.

A monomeric unit is an entity comprised of two elements. Said first element is a compound. Said second element is a polymer (or oligomer) capable of noncovalently binding, complexing or hybridizing either to the polymer or oligomeric element of a second monomeric unit or to the polymer or oligomer that makes up a binding matrix. Among others, the monomeric unit can be selected from a naturally occurring compound, a modified natural compound,

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a synthetic compound and a recombinately produced compound or combinations of such compounds.

Said compound may be an analyte specific moiety that is capable of recognizing and binding to a component in a biological system *in vivo* or *in vitro*. A biological system can be comprised of cells, cellular components, viruses, viral components, circulating material, extracellular binding matrices or combinations thereof. The compound could be naturally occurring, a modified natural compound, a synthetic compound or a recombinant product. It could be a polyclonal or monoclonal antibody, complete protein chains or f(ab) fragments, from human or other species; it could be a lymphokine, cytokine, hormone (e.g., insulin), or growth factor (e.g., erythropoietin) or a cellular matrix protein (e.g. fibronectin); it could be a ligand, vector, bacterium, or virus; it could be a monosaccharide, oligosaccharide, polysaccharide, polynucleotide, protein, or lipid.

The polymers can be attached to the compounds either covalently or noncovalently. The compounds could be covalently attached to the polymers through conjugation of reactive groups on the compound and the polymer. Either the compound or the polymer or both could be chemically modified such that conjugation could be facilitated. Either the compound or the

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polymer could be modified such that a ligand such as biotin could be introduced to one and a receptor for the ligand such as avidin introduced to the other.

It is preferred that the polynucleotide segment that is attached to a given compound does not bind to itself or hybridize together or is not substantially self-complementary. In the multimeric construct, the component could be homogeneous or heterogeneous, as long as the polymer segment on the homogeneous component or heterogeneous mixture or compounds can bind or hybridize to the same binding polymer or polynucleotide in the binding matrix.

Polymers that are attached to the compounds to form the monomeric units may be selected from the same group of polymers that comprise the binding matrices with the proviso that they should be able to bind together noncovalently.

The binding matrix is an entity comprised of a linear or branched polymeric compound that has more than one portion of a linear segment that is capable of noncovalent binding to a linear segment of a polymer of a monomeric unit.

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The polymer attached to the compound and the polymer of the binding matrix could bind to each other noncovalently through either ionic

interactions, hydrogen bonding, complementarity or polar interactions, including dipole-dipole interactions.

When the binding is through ionic interaction, if the monomeric unit contains polycationic segments, then the corresponding binding matrix should have polyanionic segments. If the monomeric unit has polyanionic segments, then the corresponding binding matrix should contain polycationic segments.

Examples of positively charged polymers could be protamine or polylysine; soluble DEAE (diethylaminoethyl) cellulose, or DEAE dextran (a branched polysaccharide).

Examples of negatively charged polymers are techoic acids (polymeric chains of glycerol or ribitol molecules linked to each other by phosphodiester bridges), polyglutamic acid, carboxymethyl cellulose, dextran sulfate (a branched polysaccharide with 3 negatively charged sulfate groups), and polyacrylic acid.

When the binding is through hydrogen bonding or complementarity, if the monomeric unit has a polynucleotide sequence attached, the corresponding binding matrix should have the complementary nucleic acid sequence.

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Binding matrix polymers preferentially have net ionic charges or sufficient polarity to be soluble and have the capability of noncovalent binding to another polymer of opposite polarity, charge, or complementarity. Such a polymer could be single or double stranded polynucleotide, RNA or DNA, modified or unmodified; polynucleic acid analogs or any other synthetic polymer that exhibits such properties.

Double stranded nucleic acid can also act as a polyanionic binding matrix. In this case the monomeric unit is attached to a polycationic entity such as polylysine or polyamine.

Another way of constructing such complexes is through protein-nucleic acid interactions. Polypeptides that exhibit high affinity levels for nucleic acids can be attached to desirable compounds to form monomeric units that can then be complexed together by binding to a nucleic acid polymer. The sequence of the nucleic acid polymer can be made up of multimers of binding sequences in the cases where the monomeric units are derived from sequence specific binding proteins such as the HIV TAR protein. However, the choice of the sequence of the nucleic acid polymer can be completely unrestricted in cases where the monomeric units are derived from sequence independent DNA binding proteins such as histone.

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One can optimize a given multicomplex compound by adjusting the number of monomeric unites in a given binding matrix such that one obtains the maximum number of compounds on a given binding matrix, while maintaining solubility and avoiding stearic hindrance to assure maximal functioning of the multicomplex construct.

When the binding of a monomeric unit to the binding matrix is through ionic interaction of two oppositely charged polymers, the ratio of the monomeric unit to the binding matrix has to be adjusted such that the net charge or the charge distribution of the multicomplex construct is sufficient to maintain solubility.

Such multimeric complexes are formed by introducing a polymer to an individual compound that can bind either to another polymer and/or can bind to a polymer of another compound. In the case of polynucleotides, the binding could be through complementary sequences. The polymers could be homopolymers or heteropolymers, sufficient in length to form a stable bond. In a stable bond formed by polynucleotides, the polymer could be from approximately 5 to several thousand nucleotides in length.

One aspect of these multicomplex units is the formation of complexes with high affinity for the target entity. A multi-antibody complex of this invention will exhibit a much higher avidity for the target antigen than a single

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antibody. Such a complex will be useful therapeutically and for *in vitro* diagnosis. *In vivo* such complexes could be used as more effective immunologic reagents, including antiviral, antibacterial and antitumor agents. In the case of *in vivo* use of such a multimeric complex, the preferred polymers are polynucleotides or modified polynucleotides since nucleic acids are better tolerated immunologically. For *in vitro* diagnostics, such multicomplexes could be used to develop more sensitive assay systems. The sensitivity of any diagnostic system depends on two factors, the sensitivity of the signal and the affinity between the analyte and analyte specific moiety. If the affinity is not high enough there could be practical or theoretical limits as to how much one could increase saturated binding in the system with the target entity.

Furthermore, such complexes could be used for efficient gene transformation both *in vivo* or *in vitro* (as discussed in the disclosure).

A certain concentration of the binding partner is required in order to obtain a certain level of binding *in vivo* as well as *in vitro*. A multimeric complex of biological binding elements, which upon binding to a cell can trigger biological effects in the cell, would have a much higher binding affinity to a target cell than the corresponding monomeric unit. Consequently much lower quantities of such a multicomplex compared to the monomeric unit would be needed to achieve the same physiological effect. Examples of such

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biological complexes are hormones, cytokines, lymphokines, growth factors, ligands. A multicomplex of insulin could be useful in that manner in diabetic treatment

In addition to being used to form more potent biological effectors, multimeric complexes or polymeric units of this invention can be used to form multimeric complexes or polymeric units of compounds which bind to etiological agents, such as viruses, bacteria and fungi, or to toxic compounds. These binding compounds could be polyclonal or monoclonal antibodies, complete protein chains or F(ab) fragments, from human or other species; or the receptor protein of the etiological agent or toxic compound. The binding of such polymers or complexes to the target is stronger than the binding of the monomers and these polymers or complexes can recognize and bind to low concentrations of the etiological agent or the toxic compound. These compositions can be applied, therefore, for more effective therapeutic use against infection and toxicity. These products can be administered to patients *in vivo* or could be used *ex vivo* for neutralization of potentially infected or toxic blood.

In preparing such complexes, one would modify a compound, such that the binding of the compound does not interfere with its biological function or effects. The preferred attachment of reactive groups or oligomers or polymers covalently or through a complex would be via non-disruptive

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chemistry. Binding is through reactive groups in the compound that are not within the active site, binding site or functional groups and binding is such as to allow maximal freedom with the least amount of disruption to the molecule.

If desired, the spacing of the monomeric units can be predetermined by defining the nature of the region that the monomeric units are bound to the matrix to optimize their spacing so as to provide proper co-operative binding and also to reduce potential steric hindrance. An example of this type of disposition of the monomeric units is shown in Figure 23 from Example 18 where each monomeric unit has been joined to a specific unique sequence that is complimentary to different portions of the M13 binding matrix.

These multicomplex compounds could further contain many other entities as ligands, receptors, chemical modifications that either enhance their biological function, increase their solubility, provide further cooperative overall binding or provide capability to bind to desired cells *in vitro* and *in vivo*. Thus another aspect of this invention is the composition, described above, further comprising an entity attached to the binding matrix. Such an entity can comprise a ligand or a compound which increases the binding of the binding matrix. Examples of such entities are the cellular matrix proteins (fibronectin), lectins, polysaccharides, and polycationic polymers such as polylysine and histones.

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Any of the above-described compositions can be formulated as homogeneous forms or compositions or heterogeneous forms or compositions.

The above-described multimeric complex composition (and its various embodiments) can be usefully employed in a process for delivering a cell effector to a cell. In such a process one would provide the multimeric complex composition wherein the monomeric unit of the composition comprises a cell effector and administer the composition either in vivo or ex vivo. In addition the multimeric complex composition can be employed in a process for delivering a gene or a gene fragment to a cell. Here, one would provide the multimeric complex composition wherein the monomeric unit comprises the gene or gene fragment to be delivered and would administer such composition either in vivo or ex vivo as the case may be.

Another useful multimeric composition comprises more than one component attached to a charged polymer. The charged polymer is selected from a polycationic polymer, a polyionic polymer, a polynucleotide, a modified polynucleotide and a polynucleotide analog as well as combinations of the foregoing. Such a component can comprise a protein, e.g., an antibody (polyclonal or monoclonal), an $F(ab')_2$ fragment or both. The antibody can be further complexed with a target comprising an enzyme.

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4. Intron Inactivation

The present invention provides (1) a universal composition for conditional nucleic acid processing by the introduction of a processing element into a nucleic acid sequence produced from a construct introduced into a cell. Said produced nucleic acid is processed in a compatible cell, i.e., a cell capable of processing RNA by removal of the processing element. Said RNA is not processed in an incompatible cell, i.e., a cell capable of processing RNA by removal of the processing element and (2) a binary biological function in which a single nucleic acid construct bearing at least two operons or transcriptional units non-native to a cell when introduced into said cell results in the protein gene product of one of the operons impacting the the protein gene product(s).

The present invention provides a novel method and constructs for capability for the conditional inactivation of a gene by the use of a non-native, or heterologous, processing element which only permits gene expression in compatible cells. The method utilizes the introduction of a heterologous processing element into the coding region of a desired gene resulting in inactivation of the gene when present in a non-compatible cell. The intron can be inserted at a number sites in most genes. The heterologous processing element carries no flanking sequences, and thus introduces no additional sequences upon insertion. In a preferred embodiment, the gene

product either is absent or inactive in an incompatible cell, but when introduced into a compatible cell yields a functional mRNA molecule which, upon translation, the gene yields an unaltered protein.

Among the significant embodiments is a nucleic acid construct which when introduced into a cell expresses a non native polymerase, the polymerase being capable of producing more than one copy of a nucleic acid sequence from the construct. This construct can further comprise a recognition site for the non native polymerase. Such a recognition site can be complementary to a primer for the non native polymerase. The primer preferably comprises transfer RNA (tRNA).

In certain embodiments the non native polymerase comprises a member selected from DNA polymerase, RNA polymerase and reverse transcriptase as well as any combination of the foregoing enzymes. The RNA polymerase preferably comprises a bacteriophage RNA polymerase, e.g., T3, T7, and SP6, or combinations thereof. Furthermore, the above-described construct can comprise a promoter for the RNA polymerase.

The nucleic acid produced from the construct can take a number of forms including but not limited to DNA, RNA, a DNA-RNA hybrid and a DNA-RNA chimera, or combinations thereof. The DNA or RNA can comprise sense or antisense, or both.

Another significant aspect of this invention concerns a nucleic acid construct which when introduced into a cell produces a nucleic acid product comprising a non native processing element which when in a compatible cell, the processing element is substantially removed during processing. The processing element can comprise an RNA processing element including but not limited to an intron, a polyadenylation signal and a capping element, or combinations of the foregoing.

The nucleic acid product can be single stranded and it can comprise any of antisense RNA, antisense DNA, sense RNA, sense DNA, a ribozyme and a protein binding nucleic acid sequence, as well as combinations of any of these. The protein binding nucleic acid sequence preferably comprises a decoy that binds a protein required for viral assembly or viral replication.

Also provided by this invention is a process for selectively expressing a nucleic acid product in a cell, the product being such that further processing is required for its functioning. The process comprises as its first step providing a nucleic acid construct which when introduced into a cell produces a nucleic acid product comprising a non-native processing element, which when in a compatible cell, the processing element being substantially removed during processing. The second step comprises introducing this construct into the cell. The processing element, e.g., an RNA processing

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element, the nucleic acid product and the steps of introducing the construct in vivo and ex vivo are all as described previously. Significantly, in this process, the construct can be introduced into a biological system containing the cell. This biological system can comprise, an organism, an organ, a tissue and a culture (cell or tissue) as well as combinations of these.

The present invention provides a universal method for utilizing processing elements, including heterologous elements, for conditional gene inactivation. Rather than a restriction enzyme site, the frequently occurring sequence (C/A)AGG post splice junction sequence is used as the insertion site. This site results from the consensus sequence resulting from an excision of an intron. The consensus splice sequence for splice donors is (C/A)AG*GU and the consensus sequence for splice acceptors is (U/C)_nN(C/U)AG*G where * represents the splice site (Mount 1982 Nucl. Acids Res. **10**, 459). The frequent occurrence of this sequence provides numerous potential sites for the insertion of processing elements. Insertion at any of these sites in a gene coding region should not affect subsequent removal of the processing element in a compatible cell. Proteins produced from processed mRNA should demonstrate no change in amino sequence or enzyme activity since only processing element sequences free of flanking exon sequences are introduced thereby allowing the processing event to regenerate the original coding sequence.

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Furthermore, the site of insertion for a processing element does not appear to affect gene expression. Mayeda and Oshima (1990 Nucl. Acids Res. **18**: 4671, incorporated by reference) showed that a native intron, isolated as a restriction fragment of DNA containing the β -globin intron with the conserved bases of the 3' end of the donor exon attached, could be introduced into various sites of a cDNA copy of β -globin and subsequently be spliced out normally, irrespective of intron location in the β -globin coding sequence. This is consistent with the consensus sequences that have been identified for splice donors and splice acceptors and that there are no particular requirements for a specific sequence at the 5' end of the acceptor exon.

It is possible that insertion of a heterologous processing element may not in all cases inactivate a gene when present in an incompatible cell. Although splicing has been observed in procaryotic systems for bacteriophage T4 (Chu et al. 1984 Proc. Nat. Acad. Sci. USA **81**: 3049, incorporated by reference), it is in this case due to a self-splicing intron (Chu et al., 1985 J. Biol. Chem. **260**: 10680, incorporated by reference) and thus independent of processes employed in compatible cells. Therefore, in a procaryotic environment, the intron should remain in the mRNA as long as a self-splicing intron is not used. In addition, if the number of bases in the intron is a multiple of 3, the reading frame remains the same and a fusion protein with additional amino

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acids derived from the intron sequence could potentially be produced. These extra bases may or may not change the activity of the target protein depending upon the nature of the extra amino acids and the insertion site within the protein coding sequence. A preferred mode of inactivation is the use a heterologous processing element that introduces a frame shift mutation and/or a stop codon(s).

The present invention also provides for the introduction of genes not native to a cell into said cell wherein the protein products of such introduced gene(s) interact with and impact other proteins produced from introduced non-native gene(s).

The non-native protein gene products resulting from an introduced non-native gene(s) can impact another non-native protein by a variety of processes including polymerization; activation; facilitating transport; competitive inhibition; allosteric interaction; chemical modification including phosphorylation, dephosphorylation, methylation, demethylation, proteolysis, nuclease activity, glycosylation; and others.

Non-native genes can be introduced into cells as RNA, DNA or both DNA and RNA. Non-native genes can be introduced into a cell linked together on a single nucleic acid construct or introduced separately on distinct constructs.

Figure 1. The effect of the concentration of the *Agrobacterium* strain on the transformation efficiency of *Agrobacterium* strain 102. The *Agrobacterium* strain 102 was cultured in YEA medium for 24 h at 28°C. The cell concentration was adjusted to 1.0 × 10⁸ cells/ml. The cell suspension was mixed with the cell suspension of the *Agrobacterium* strain 102 at a ratio of 1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, 1:8, 1:9, 1:10, 1:11, 1:12, 1:13, 1:14, 1:15, 1:16, 1:17, 1:18, 1:19, 1:20, 1:21, 1:22, 1:23, 1:24, 1:25, 1:26, 1:27, 1:28, 1:29, 1:30, 1:31, 1:32, 1:33, 1:34, 1:35, 1:36, 1:37, 1:38, 1:39, 1:40, 1:41, 1:42, 1:43, 1:44, 1:45, 1:46, 1:47, 1:48, 1:49, 1:50, 1:51, 1:52, 1:53, 1:54, 1:55, 1:56, 1:57, 1:58, 1:59, 1:60, 1:61, 1:62, 1:63, 1:64, 1:65, 1:66, 1:67, 1:68, 1:69, 1:70, 1:71, 1:72, 1:73, 1:74, 1:75, 1:76, 1:77, 1:78, 1:79, 1:80, 1:81, 1:82, 1:83, 1:84, 1:85, 1:86, 1:87, 1:88, 1:89, 1:90, 1:91, 1:92, 1:93, 1:94, 1:95, 1:96, 1:97, 1:98, 1:99, 1:100, 1:101, 1:102, 1:103, 1:104, 1:105, 1:106, 1:107, 1:108, 1:109, 1:110, 1:111, 1:112, 1:113, 1:114, 1:115, 1:116, 1:117, 1:118, 1:119, 1:120, 1:121, 1:122, 1:123, 1:124, 1:125, 1:126, 1:127, 1:128, 1:129, 1:130, 1:131, 1:132, 1:133, 1:134, 1:135, 1:136, 1:137, 1:138, 1:139, 1:140, 1:141, 1:142, 1:143, 1:144, 1:145, 1:146, 1:147, 1:148, 1:149, 1:150, 1:151, 1:152, 1:153, 1:154, 1:155, 1:156, 1:157, 1:158, 1:159, 1:160, 1:161, 1:162, 1:163, 1:164, 1:165, 1:166, 1:167, 1:168, 1:169, 1:170, 1:171, 1:172, 1:173, 1:174, 1:175, 1:176, 1:177, 1:178, 1:179, 1:180, 1:181, 1:182, 1:183, 1:184, 1:185, 1:186, 1:187, 1:188, 1:189, 1:190, 1:191, 1:192, 1:193, 1:194, 1:195, 1:196, 1:197, 1:198, 1:199, 1:200, 1:201, 1:202, 1:203, 1:204, 1:205, 1:206, 1:207, 1:208, 1:209, 1:210, 1:211, 1:212, 1:213, 1:214, 1:215, 1:216, 1:217, 1:218, 1:219, 1:220, 1:221, 1:222, 1:223, 1:224, 1:225, 1:226, 1:227, 1:228, 1:229, 1:230, 1:231, 1:232, 1:233, 1:234, 1:235, 1:236, 1:237, 1:238, 1:239, 1:240, 1:241, 1:242, 1:243, 1:244, 1:245, 1:246, 1:247, 1:248, 1:249, 1:250, 1:251, 1:252, 1:253, 1:254, 1:255, 1:256, 1:257, 1:258, 1:259, 1:260, 1:261, 1:262, 1:263, 1:264, 1:265, 1:266, 1:267, 1:268, 1:269, 1:270, 1:271, 1:272, 1:273, 1:274, 1:275, 1:276, 1:277, 1:278, 1:279, 1:280, 1:281, 1:282, 1:283, 1:284, 1:285, 1:286, 1:287, 1:288, 1:289, 1:290, 1:291, 1:292, 1:293, 1:294, 1:295, 1:296, 1:297, 1:298, 1:299, 1:300, 1:301, 1:302, 1:303, 1:304, 1:305, 1:306, 1:307, 1:308, 1:309, 1:310, 1:311, 1:312, 1:313, 1:314, 1:315, 1:316, 1:317, 1:318, 1:319, 1:320, 1:321, 1:322, 1:323, 1:324, 1:325, 1:326, 1:327, 1:328, 1:329, 1:330, 1:331, 1:332, 1:333, 1:334, 1:335, 1:336, 1:337, 1:338, 1:339, 1:340, 1:341, 1:342, 1:343, 1:344, 1:345, 1:346, 1:347, 1:348, 1:349, 1:350, 1:351, 1:352, 1:353, 1:354, 1:355, 1:356, 1:357, 1:358, 1:359, 1:360, 1:361, 1:362, 1:363, 1:364, 1:365, 1:366, 1:367, 1:368, 1:369, 1:370, 1:371, 1:372, 1:373, 1:374, 1:375, 1:376, 1:377, 1:378, 1:379, 1:380, 1:381, 1:382, 1:383, 1:384, 1:385, 1:386, 1:387, 1:388, 1:389, 1:390, 1:391, 1:392, 1:393, 1:394, 1:395, 1:396, 1:397, 1:398, 1:399, 1:400, 1:401, 1:402, 1:403, 1:404, 1:405, 1:406, 1:407, 1:408, 1:409, 1:410, 1:411, 1:412, 1:413, 1:414, 1:415, 1:416, 1:417, 1:418, 1:419, 1:420, 1:421, 1:422, 1:423, 1:424, 1:425, 1:426, 1:427, 1:428, 1:429, 1:430, 1:431, 1:432, 1:433, 1:434, 1:435, 1:436, 1:437, 1:438, 1:439, 1:440, 1:441, 1:442, 1:443, 1:444, 1:445, 1:446, 1:447, 1:448, 1:449, 1:450, 1:451, 1:452, 1:453, 1:454, 1:455, 1:456, 1:457, 1:458, 1:459, 1:460, 1:461, 1:462, 1:463, 1:464, 1:465, 1:466, 1:467, 1:468, 1:469, 1:470, 1:471, 1:472, 1:473, 1:474, 1:475, 1:476, 1:477, 1:478, 1:479, 1:480, 1:481, 1:482, 1:483, 1:484, 1:485, 1:486, 1:487, 1:488, 1:489, 1:490, 1:491, 1:492, 1:493, 1:494, 1:495, 1:496, 1:497, 1:498, 1:499, 1:500, 1:501, 1:502, 1:503, 1:504, 1:505, 1:506, 1:507, 1:508, 1:509, 1:510, 1:511, 1:512, 1:513, 1:514, 1:515, 1:516, 1:517, 1:518, 1:519, 1:520, 1:521, 1:522, 1:523, 1:524, 1:525, 1:526, 1:527, 1:528, 1:529, 1:530, 1:531, 1:532, 1:533, 1:534, 1:535, 1:536, 1:537, 1:538, 1:539, 1:540, 1:541, 1:542, 1:543, 1:544, 1:545, 1:546, 1:547, 1:548, 1:549, 1:550, 1:551, 1:552, 1:553, 1:554, 1:555, 1:556, 1:557, 1:558, 1:559, 1:560, 1:561, 1:562, 1:563, 1:564, 1:565, 1:566, 1:567, 1:568, 1:569, 1:570, 1:571, 1:572, 1:573, 1:574, 1:575, 1:576, 1:577, 1:578, 1:579, 1:580, 1:581, 1:58

a) This invention has utility for the conditional inactivation of genes when such genes would be lethal to the host cell or when such genes present in a host cell introduce a danger. Thus, genes which would be impossible to clone, such as those which code for enzymes which destroy bacterial cell walls, can be inactivated by intron insertion and thus cloned in this form in a bacterium. Genes coding for toxic products, including tetanus toxin, risin, pseudomonas toxin, *E. coli* enterotoxins, cholera toxin and other plant, animal and microbial toxins, can be inactivated and maintained stably and safely in an incompatible cell and activated to produce an unaltered gene product in a compatible cell. This has special application to cell killing gene therapy.

b) The present invention provides utility for the inactivation, in incompatible cells, of the expression of polymerase catalysts whose expression can be realized in compatible cells. This has application to expression of a variety of gene products, either RNA or protein, under control of promoters of a variety of polymerases. Polymerases, native and non-native to the cell, that could be used in this way include RNA polymerases from T3, T7 and SP6.

c) This invention provides for normally incompatible genes to be cloned together on the same nucleic acid construct . For example, a single construct can be designed containing sequences for the production of T7 promoter directed transcript(s) of choice and T7 RNA polymerase. The ability to clone such genes on the same nucleic acid construct rather than as separate constructs provides the following benefits:

- i) The efficiency of cotransfection of the two genes is 100%.
- ii) In the case of T7 RNA polymerase and a nucleic acid sequence for T7-directed transcript of choice, the entire functional unit is sufficiently compact that it can be cloned into a vector which can only accept inserts below a certain size limit as, for example, adeno associated virus which can only accept inserts of 4.7 kilobases or below and remain functional (Muzyczka 1992 in Current Topics in Microbiology and Immunology, Springer Verlag, Heidelberg, 158;97, incorporated by reference)

d) Another application of this invention provides for the interaction of non-native gene or its protein products in a cell where the interaction of the introduced genes and/or their gene products can yield useful intracellular processes for gene therapy applications.

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In an application of the present invention, an intron is introduced into the coding sequence of T7 RNA polymerase in a construct that also contains a T7 promoter directing the transcription of a useful gene product. As discussed earlier, the use of T7 polymerase for synthesis of a gene under control of a T7 promoter has been accomplished in compatible cells, but always in the context of placing the two entities on separate constructs, *i.e.*, the T7 RNA polymerase and the gene under the control of a T7 promoter are used as a two-part system. The present invention (see Examples) describes the conditional inactivation of a gene (that normally does not contain a processing element) by the precise introduction of an intron between the last two G's of a site that has the post splice junction sequence (C/A)AGG. The introduction of an intron into sites with this sequence creates a functional splice donor and a functional splice acceptor. Therefore, a construct with this modification should lack any expression of T7 RNA polymerase in an *E. coli* cell, but the normal coding sequence can be restored from transcripts after introduction into a compatible cell. This allows the construction of a single construct that contains both the T7 RNA polymerase and, for example, antisense directed by a T7 promoter, with lethality to an incompatible cell being avoided by introduction of the heterologous processing element into the polymerase coding sequence. In a compatible cell, normal expression of the polymerase will occur but lethality should be negated by the nature of its environment. First, the autocatalytic cascade, due to transcription around

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	1980	1981	1982	1983	1984	1985	1986	1987	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022	2023	2024	2025	2026	2027	2028	2029	2030	2031	2032	2033	2034	2035	2036	2037	2038	2039	2040	2041	2042	2043	2044	2045	2046	2047	2048	2049	2050	2051	2052	2053	2054	2055	2056	2057	2058	2059	2060	2061	2062	2063	2064	2065	2066	2067	2068	2069	2070	2071	2072	2073	2074	2075	2076	2077	2078	2079	2080	2081	2082	2083	2084	2085	2086	2087	2088	2089	2090	2091	2092	2093	2094	2095	2096	2097	2098	2099	2100	2101	2102	2103	2104	2105	2106	2107	2108	2109	2110	2111	2112	2113	2114	2115	2116	2117	2118	2119	2120	2121	2122	2123	2124	2125	2126	2127	2128	2129	2130	2131	2132	2133	2134	2135	2136	2137	2138	2139	2140	2141	2142	2143	2144	2145	2146	2147	2148	2149	2150	2151	2152	2153	2154	2155	2156	2157	2158	2159	2160	2161	2162	2163	2164	2165	2166	2167	2168	2169	2170	2171	2172	2173	2174	2175	2176	2177	2178	2179	2180	2181	2182	2183	2184	2185	2186	2187	2188	2189	2190	2191	2192	2193	2194	2195	2196	2197	2198	2199	2200	2201	2202	2203	2204	2205	2206	2207	2208	2209	2210	2211	2212	2213	2214	2215	2216	2217	2218	2219	2220	2221	2222	2223	2224	2225	2226	2227	2228	2229	2230	2231	2232	2233	2234	2235	2236	2237	2238	2239	2240	2241	2242	2243	2244	2245	2246	2247	2248	2249	2250	2251	2252	2253	2254	2255	2256	2257	2258	2259	2260	2261	2262	2263	2264	2265	2266	2267	2268	2269	2270	2271	2272	2273	2274	2275	2276	2277	2278	2279	2280	2281	2282	2283	2284	2285	2286	2287	2288	2289	2290	2291	2292	2293	2294	2295	2296	2297	2298	2299	2300	2301	2302	2303	2304	2305	2306	2307	2308	2309	2310	2311	2312	2313	2314	2315	2316	2317	2318	2319	2320	2321	2322	2323	2324	2325	2326	2327	2328	2329	2330	2331	2332	2333	2334	2335	2336	2337	2338	2339	2340	2341	2342	2343	2344	2345	2346	2347	2348	2349	2350	2351	2352	2353	2354	2355	2356	2357	2358	2359	2360	2361	2362	2363	2364	2365	2366	2367	2368	2369	2370	2371	2372	2373	2374	2375	2376	2377	2378	2379	2380	2381	2382	2383	2384	2385	2386	2387	2388	2389	2390	2391	2392	2393	2394	2395	2396	2397	2398	2399	2400	2401	2402	2403	2404	2405	2406	2407	2408	2409	2410	2411	2412	2413	2414	2415	2416	2417	2418	2419	2420	2421	2422	2423	2424	2425	2426	2427	2428	2429	2430	2431	2432	2
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5. Hairpin Construct

The introduction of genetic material into cells can be done by two methods. One method is the exogenous application of nucleic acids which act directly on cellular processes but which themselves are unable to replicate or produce any nucleic acid. The intracellular concentrations of these molecules that must be achieved in order to affect cellular processes is dependent on the exogenous supply. Another method for nucleic acid delivery is the introduction into cells of Primary Nucleic Acid Constructs which themselves do not act on cellular processes but which produce single stranded nucleic acid in the cell which acts on cellular processes. In this case the introduced Primary Nucleic Acid Construct can integrate into cellular nucleic acid or it

can exist in an extrachromosomal state, and it can propagate copies of itself in either the integrated or the extrachromosomal state. The nucleic acid construct can produce, from promoter sequences in the Primary Nucleic Acid Construct, single stranded nucleic acids which affect cellular processes of gene expression and gene replication. Such nucleic acids include antisense nucleic acids, sense nucleic acids and transcripts that can be translated into protein. The intracellular concentrations of such nucleic acids are limited to promoter-dependent synthesis.

Definitions:

Primary Nucleic Acid Construct. A composition consisting of nucleic acid which in a cell propagates Production Centers.

Production Center. A nucleic acid molecule derived from a Primary Nucleic Acid Construct which in a cell is able to propagate other Production Centers or to produce single stranded nucleic acid. As used herein, the term production center is intended to cover secondary nucleic acid components which can be produced from a primary nucleic acid construct. Also covered are a tertiary nucleic acid component which could be produced from the secondary nucleic acid component, as well as any nucleic acid product which may be produced from the secondary nucleic acid component.

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Propagation. The generation or formation of a Production Center from a Primary Nucleic Acid Construct or the generation or formation of a Production Center from another Production Center. However, production centers cannot produce a Primary Nucleic Acid Construct.

Production. The generation of a single stranded nucleic molecules from a Production Center.

Inherent Cellular Systems. Cellular processes and components present in cells which can be utilized for the Production and Propagation as well as the function of single stranded Nucleic Acid Products. Such processes and components can be native to the cell, or be introduced to the cell by artificial means or by infection by, for example, a virus.

The effectiveness of single stranded nucleic acids produced from Primary Nucleic Acid Constructs is dependent on their concentration, the stability and the duration of production in the cell. Current methods for achieving intracellular concentrations are limited by a dependence on promoter directed synthesis.

The present invention provides a novel composition construct and method whereby single stranded nucleic acid is produced in the cell from templates which are formed in the cell and derived from Primary Nucleic Acid

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Constructs in said cell. This invention further provides for a Primary Nucleic Acid Construct which, when introduced into a cell Propagates one or more Production Centers each of which in the cell is capable of Production of single stranded nucleic acid product.

One aspect of the present invention provides a means to attain high intracellular levels of single stranded nucleic acid through amplification. Such amplification occurs by the Propagation from a Primary Nucleic Acid Construct of more than one Production Center and from each Production Center one or more single stranded nucleic acids. However, Production Centers are not capable of producing Primary Nucleic Acid Constructs.

Thus, a significant embodiment of this invention concerns a composition comprising a primary nucleic acid component which upon introduction into a cell produces a secondary nucleic acid component which is capable of producing a nucleic acid product, or a tertiary nucleic acid component, or both. The secondary and tertiary nucleic acid components and the nucleic acid product are incapable of producing the primary nucleic acid component. In this composition the cell can of course be eukaryotic or prokaryotic.

In the present composition, the primary nucleic acid component can comprise a nucleic acid, a nucleic acid construct, a nucleic acid conjugate, a virus, a viral fragement, a viral vector, a viroid, a phage, a phage vector, a plasmid, a

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plasmid vector, a bacterium, and a bacterial fragment or combinations of any of these.

Primary Nucleic Acid Constructs consist of single or double stranded nucleic acid (or even partially double stranded) or composed of both single and double stranded nucleic acid, and the nucleic acid can be RNA, DNA or a combination of RNA and DNA. The nucleic acid can be unmodified or it can be modified to provide desirable properties. For example, modified bases can be incorporated to provide nuclease resistance, interaction with Inherent Cellular Systems, cellular localization and other properties for nucleic acid constructs as described in this disclosure. Furthermore, the primary nucleic acid component can comprise nucleic acid analogs which likewise can be used in combination with DNA, RNA, or both.

Primary Nucleic Acid Constructs can reside in the cell integrated into chromosomal DNA or as extrachromosomal entities. The Primary Nucleic Acid Construct, as an integral part of a chromosome, can be replicated concomitant with chromosomal DNA during cell division processes or it can be replicated as part of an extrachromosomal element containing DNA replication elements, such as sequences for origin of replication and others.

Primary Nucleic Acid Constructs contain sequence information for the Propagation of Production Centers and for the subsequent Production of

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single stranded product. Thus, for this purpose, a variety of desirable elements can be encoded in a Primary Nucleic Acid Construct. Production Centers and Primary Nucleic Acid Constructs may contain one or all of these elements. These include regulatory elements such as promoters and enhancers; primer binding sites; processing elements such as intron sequences, poly A sequences, sequences specifying capping and termination sequences; sequences specifying cellular localization signal sequences with affinity for cellular proteins. Primary Nucleic Acid Constructs can also contain sequences for the synthesis of proteins which act to propagate Production Centers. For example, sequences for a nucleic acid polymerase which acts to propagate a Production Center can be present in a Primary Nucleic Acid Construct (See Example 20 of this patent).

Primary Nucleic Acid Constructs can propagate Production Centers through the activity of nucleic acid polymerizing catalysts present as Inherent Cellular Systems. Production Centers can be RNA, DNA or a combination of RNA and DNA. They can be single stranded, double stranded or contain both single and double stranded regions. Production Centers can propagate other Production Centers and/or produce single stranded nucleic acid product with biological activity directly or through the activity of Inherent Cellular Systems.

Production Centers can produce a variety of single stranded nucleic acids such as antisense RNA sequences, antisense DNA sequences, ribozyme sequences and mRNAs which can be translated into proteins can all be produced. Desirable properties to enhance biological activity can also be incorporated. Thus, RNA processing signals, sequences specifying cellular location, sequences for binding cellular proteins and other functions can be incorporated into single stranded nucleic acids products.

As production centers, the secondary nucleic acid component and the tertiary nucleic acid component (as well as other subsequent components, e.g., a quaternary nucleic acid component, can comprise DNA, RNA, a DNA-RNA hybrid, and a DNA-RNA chimera or a combination of the foregoing.

When the above-described compositions further comprise a signal processing sequence, such sequences can be selected from a promoter, an initiator, a terminator, an intron, and a cellular localization element or a combination of these. Such signal processing sequences can be contained in any of the elements of the composition including those selected from the primary nucleic acid component, the secondary nucleic acid component, the nucleic acid product and the tertiary nucleic acid or a combination of these. The nucleic acid product can of course be single stranded as well as comprising antisense RNA, antisense DNA, a ribozyme and a protein binding nucleic acid sequence or combinations of these. Preferred as a protein binding nucleic

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acid sequence is a decoy that binds a protein required for viral assembly or viral replication.

In these above-described compositions, production of any component or nucleic acid product can be mediated by a vector, preferred vectors comprise viral vectors, phage vectors, plasmid vectors, as well as combinations of these.

The present composition can be incorporated into a cell which is eukaryotic or prokaryotic. The composition can be introduced either in vivo or ex vivo into such a cell.

Also contemplated by the present invention are production centers including the secondary or tertiary nucleic acid components or the nucleic acid product which can be produced from the composition.

The Propagation of Production Centers from Primary Nucleic Acid Constructs, the Propagation of Production Centers from other Production Centers and the Production of single stranded nucleic acid from Production Centers can proceed by a variety of processes which derive from sequences present in these structures (as described above) and from Inherent Cellular Systems. Inherent Cellular Systems involved in these processes include RNA

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polymerases, RNA processing enzymes, DNA polymerases, Reverse Transcriptases, Ribonuclease H, endonucleases, exonucleases including ribozymes, enzymes involved in nucleic acid repair, nucleic acid ligases, cellular nucleic acids acting as primers, and entities involved in nucleic acid replication, transcription, translation, localization of nucleic acid in the cell, transport of nucleic acid, integration of nucleic acid into cellular nucleic acid and others.

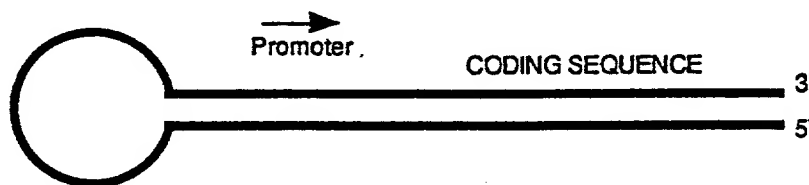
Elements for Propagation and Production include: 1) single or multiple promoters, 2) self priming processes, 3) one or more primer binding sites, and 4) multiple priming.

1) Promoters for Propagation and Production can be present in one or more copies in a Production Center or in a Primary Nucleic Acid Construct. Such promoter sequences can be present in a preexisting and functional form, as, for example, in a double stranded DNA Primary Nucleic Acid Construct introduced into a cell. Functional promoter sequences can also form subsequent to introduction of a Primary Nucleic Acid Construct into a cell. For example, a single stranded RNA Primary Nucleic Acid Construct containing promoter sequences which are non-functional (since they are present as single stranded ribonucleic acid) can be converted to functional promoter sequences by propagation in the cell to a double stranded DNA Production Center from said Primary Nucleic Acid Construct. This

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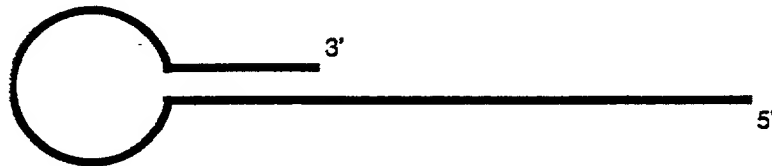
Propagation can be achieved by the presence in the Primary Nucleic Acid Construct of primer binding sites, such as the HIV primer binding site for which lysyl tRNA acts as a primer, and reverse transcriptase as an Inherent Cellular Element. The generation of double stranded DNA in this way forms a functional promoter.

Functional promoter sequences can also be generated by the formation of double stranded regions from self complementary formation in a single stranded Primary Nucleic Acid Construct. For example, the presence of both the sense and antisense sequences for a promoter and a coding sequence under its control can be present in a single stranded DNA Nucleic Acid Product or Production Center. Self hybridization of these regions of the same molecule can generate a functional promoter in the formed double stranded region of this single stranded molecule.



2) A single stranded Primary Nucleic Acid Construct can Propagate or a linear single stranded Production Center can Propagate or Produce nucleic acids by a self priming process. In this process, the 3' end of such a molecule can hybridize with complementary regions located elsewhere in the

molecule and act as a primer for the synthesis of complementary nucleic acid. For example, the 3' end of a linear single stranded RNA can act as a primer for a polymerase such as reverse transcriptase.



3) One or more primer site sequences can be included in a Primary Nucleic Acid Construct or in a Product Center. Sequences for the primer binding site of a retrovirus, such as HIV, which utilizes lysyl tRNA as a primer, can be included in one or more copies in a single stranded RNA Primary Nucleic Acid Construct or Production Center. Lysyl tRNA is supplied as an inherent cellular system. In the presence of reverse transcriptase, Propagation and Production of complementary DNA proceeds from the primer site.

4) Multiple priming processes can be utilized for Production and Propagation. For example, a double stranded Primary Nucleic Acid Construct composed of one DNA strand and one RNA strand can be acted upon by nucleases to generate limited endonucleolytic cleavage in the RNA strand. The resulting fragments can act as primers the production and propagation of DNA synthesis as catalyzed by inherent cellular processes such as reverse transcriptase.

6. U1 Antisense System

This invention provides a composition of matter comprising a nucleic acid component which when present in a cell produces a non-natural nucleic acid product, the product comprising two elements: a portion of a localizing entity and a nucleic acid of sequence. The portion of the localizing entity is preferably sufficient to permit localization of the non natural nucleic acid product. Furthermore, the portion of the localizing entity preferably comprises a cytoplasmic or nuclear localization signaling sequence.

The nucleic acid sequence of interest can comprise various forms of nucleic acid including but not limited to DNA, RNA, a DNA-RNA hybrid and a DNA-RNA chimera or combinations of these. When comprising RNA, the nucleic acid of sequence preferably comprises a nuclear localized RNA which may be complexed with protein molecules. Among such nuclear localized RNA are the so called snRNAs. Preferred as snRNA's are U1, or U2, or both.

The non natural nucleic acid product can be of course single stranded and it may comprise various members or forms including those selected from antisense RNA, antisense DNA, sense RNA, sense DNA, a ribozyme, and a protein binding nucleic acid sequence. As described elsewhere, such a protein binding nucleic acid sequence preferably comprises a decoy that

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binds a protein involved or required for viral assembly or replication. In another aspect of the present composition, the non natural nucleic acid product comprises antisense RNA or antisense DNA and the portion of the localizing entity comprises a nuclear localization signalling sequence. In yet another aspect of the composition, the non-natural nucleic acid product comprises antisense RNA or antisense DNA and the portion of the localizing entity comprises a cytoplasmic localization signalling sequence. Still yet another aspect concerns the composition wherein the non-natural nucleic acid product comprises sense RNA or sense DNA and the portion of a localizing entity comprises a cytoplasmic localization signalling sequence,

As described elsewhere the nucleic acid component can take various forms, e.g., a nucleic acid, a nucleic acid construct, a nucleic acid conjugate, a virus, or fragment, a viroid, a phage, a plasmid, a vector, a bacterium, or fragment, as well as any combination of these. Such nucleic acid can comprise DNA, RNA, a DNA-RNA hybrid and a DNA-RNA chimera and combinations thereof. The nucleic acid can be modified; the cell can be eukaryotic or prokaryotic. The production of nucleic acid product is mediated by a vector such as a viral vector, a phage vector, or a plasmid vector or such combinations.

As described elsewhere the present combination can be incorporated or delivered into a cell which can be eukaryotic or prokaryotic. Introduction into

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the cell can be ex vivo or in vivo. The present invention also contemplates biological systems (an organism, an organ, a tissue, a culture) containing the cell into which the composition has been introduced.

The present invention further contemplates a process for localizing a nucleic acid product in a eukaryotic cell. In this process, the above-described composition of matter would be provided and appropriately introduced into a eukaryotic cell or a biological system containing such cell. The characteristics of the localizing entity portion, the nucleic acid product, methods, ex vivo and in vivo introduction in this process are all as described above.

The present invention describes a method and composition for utilising snRNAs as carriers for antisense RNA while retaining the advantageous features of snRNA for nuclear localization. The present invention utilizes removal of sequences from snRNA and their replacement with desirable sequences such as antisense or sense sequences.

The correct choice of the site for replacement of a portion of the snRNA sequence should not alter the stability and nuclear reimportation features. Digestion of a clone of the human U1 operon with Bcl I and Bsp E II (Figure 41) eliminates a sequence of 49 bases involved in the formation of the A and B loops formed by U1 RNA (Figure 41). Removal of this sequence thus both

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makes room for the addition of foreign sequence and eliminates binding of some snRNP proteins thus enabling the foreign sequence to be available for antisense inhibition free of potential steric hindrance by bound proteins. Elimination of the A and B loops should still allow formation of the C and D loops which are important for maintaining the re-importation signal (Figure 41). The continued presence of this secondary structure at the 3' end as well as binding of spliceosome proteins should also have the effect of maintaining the stability of the RNA.

This invention should be applicable to other species of snRNA including U2.

U1 constructs prepared as described for this invention can be delivered to cells as all or part of nucleic acid constructs by any of several methods applicable to gene delivery.

7. Multi-Cassette Constructs

The present invention, which has application to gene therapy, is a Nucleic Acid Entity which, when introduced into a cell, directs the synthesis of more than one specific entity from a separate functional unit, or cassette. The synthesis of each product entity is initiated from its own initiator signal in a cassette. Multi-targeting can be achieved by inclusion of independent cassettes in a single Multi-Cassette Construct. The advantages of a Multi-Cassette are:

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a) Each entity is formed independently from other entities and the total number of product entities will be a summation of the products generated in the cell by each initiation site.

b) Interaction with a target by an independently generated product entity should have no effect upon the activity of other independently generated product entities.

c) An integration event that disrupts expression from one cassette should have no effect upon other cassettes in the construct.

d) Each product entity present in a construct can be directed to a different intracellular locus by use of appropriate signals for either nuclear or cytoplasmic localization. In situations where product entities acting in the nucleus are combined in the same construct with entities acting in the cytoplasm, the application of Multi-Cassette Constructs allows independent synthesis of the two entities, thereby allowing each to accumulate at its most effective site of action.

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This invention provides a nucleic acid component which upon introduction into a cell is capable of producing more than one specific nucleic acid sequence. Each such specific sequence so produced are substantially nonhomologous with each other and are either complementary with a specific portion of a single-stranded nucleic acid of interest in a cell or are capable of binding to a specific protein of interest in a cell.

In this component, the single stranded nucleic acids of interest can be part of the same polynucleotide sequence or part of different polynucleotide sequences. The single stranded nucleic acids of interest can comprise viral sequences. The present nucleic acid component can be derived or selected from any of nucleic acids, nucleic acid constructs, nucleic acid conjugates, a virus or fragment, a phage, a plasmid, a bacterium, or fragment, a vector (viral, phage, plasmid), as well as any combinations of these. The nucleic acid can comprise DNA, RNA, and nucleic acid analogs (or combinations thereof). The DNA and RNA can be modified.

In addition, the nucleic acid component can comprise either more than one promoter or more than one initiator, or both. Furthermore, the specific nucleic acid sequence products can be produced independently from either different promoters, different initiators, or combinations of both. Still further, the specific nucleic acid sequence products can be either complementary to a viral or cellular RNA or bind to a viral or cellular protein or a combination of

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such things. The complementary specific nucleic acid sequence products can be capable of acting as antisense. The viral or cellular protein can comprise a localizing protein or a decoy protein which are described elsewhere. Such localizing proteins preferably comprise a nuclear localizing protein or a cytoplasmic localizing protein. Specific nucleic acid sequence products can comprise antisense RNA, antisense DNA, a ribozyme, and a protein binding nucleic acid sequence or a combination of the foregoing.

The nucleic acid component can further comprise a means for delivering the component to a cell containing the nucleic acid of interest or the specific protein of interest. Such delivering means are known in the art as well as described elsewhere in the disclosure.

The Multi-Cassette Constructs can be prepared as RNA or DNA. The nucleic acid can be delivered to the cells as modified or unmodified nucleic acid or as modified or unmodified RNA or DNA complexed to proteins, lipids or other molecules or as modified or unmodified RNA or DNA as components of pseudo virions, bacteriophage or other viral delivery systems.

Multi-Cassette constructs can be delivered to target cells by methods commonly used for gene transfer as described in this application.

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The presence of independent synthesis units, *i.e.*, cassettes, in a Multi-Cassette Construct provides versatility for the presentation of product entities to the cell through the choice of product entities, synthesis initiator signals and other elements. A Multi-Cassette Construct can be designed to code for a variety of product entities. Thus, cassettes can be designed to code for synthesis of RNA, DNA or protein and such cassettes can be assembled in various combinations in a single Multi-Cassette Construct.

Elements can be incorporated into each cassette to regulate the independently and differentially, if desirable the synthesis, character and nature and activity of the product entity in the cell. Such elements include the type of promoter, enhancer sequences, RNA processing elements such as introns, cellular localization elements such as nuclear or cytoplasmic localization signals and poly A addition signals to provide for addition of poly-A to mRNA.

Useful product entities produced by each cassette include antisense RNA, sense RNA, ribozymes antisense DNA, nucleic acid sequences which bind protein molecules such as decoys which bind proteins required for virus replication: enzymes; toxin molecules; proteins which act in cellular localization of RNA and protein molecules; DNA polymerases; reverse transcriptases; RNA polymerases and nucleic acid sequences under control of cognate promoters for such RNA polymerases; proteins which impart viral

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resistance to a cell (such as interferons); antibodies and/or fragments thereof; proteins which arrest cell division ; proteins which localize in the cell membrane including cellular receptors for viruses, hormones, growth factors and other agents which interact at the cell surface;

Intracellular synthesis of product entities can be controlled by the choice of promoter or initiating element. Thus, a cassette can be designed which contains sequences for a product entity whose synthesis is under control of an inducible promoter providing for temporal synthesis of product entities. This provides advantages to applications wherein, for example, constant production of the product entity would have deleterious effects for the host cell or organism, but whose short term effects are beneficial. For example, induction of a product entity which arrests cell division processes can impart to the cell virus resistance where virus replication is dependent on such cellular processes. In order to restore the cellular processes at a later time, induction can be terminated. Induction can be mediated by use of promoters which can be induced by small molecules such as antibiotics, hormones and heavy metals such as zinc. Alternatively, in cases where constant production of a product entity or entities is beneficial, a promoter not subject to induction can be utilized.

Promoters can also be chosen on the basis of their efficiency. In cases where high levels of product entities are required promoters which initiate

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transcription at a high frequency can be utilized. Alternatively, when lower levels of product entities are desirable less efficient promoters can be used.

Independently synthesized product entities produced from the same Multi-Cassette Construct can act at the same target site. For example, in order to increase effectiveness, a series of antisense RNA product entities directed at a viral nucleic acid target site which demonstrates sequence variability, such as one of the highly variable regions of the nucleic acid of HIV, can be designed to include the predominantly occurring sequences encountered in the wild type HIV population.

Independently synthesized product entities produced from the same Multi-Cassette Construct can also act at separate target sites. For example, an RNA antisense transcript can be directed at mRNA coding for a particular gene product and a different antisense transcript can be directed against an mRNA coding for another gene product.

8. Virus Resistance

The present invention involves the use of agents that *in vivo* act to increase resistance to viruses by gene therapy by interfering with virus-cell interaction and thus enhancing antiviral gene therapy in the cell. The interaction of regions on viruses with specific sites on the cell surface, *i.e.*, virus-cell

interaction, and the susceptibility of extracellular virus to immunological agents provide the basis for supplemental treatment. Agents that act by these means to decrease the effective levels of virus would provide benefit for gene therapy treatments utilizing antisense.

As a supplement to gene therapy, the above agents can be administered to the patient either prior to, concurrently or after a gene therapy procedure by intramuscular, intravenous, intraperitoneal, by inhalation or other appropriate means.

Examples of agents that can interfere with the interaction of a virus and a target cell include:

- a) agents such as antibodies to viral epitopes and cellular proteins which bind viruses. An example of the latter are cellular receptors recognized by viruses, as, for instance the CD4 receptor that is recognized by HIV.
- b) agents that stimulate the production of entities that complex with viruses. These include adjuvants that enhance immunological responses which can be used as a general stimulant and viral antigens that can be used to induce a specific response;

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c) agents that bind to a target cell and compete with or otherwise slow the entry of a virus into a cell. Viral proteins, such as the gp124 protein for HIV, that are involved in cell binding could be used in this way. Antibodies to viral proteins can also act in this way.

In the practice of this invention, additional enhancement can be achieved by the further administration of small molecules such as protease inhibitors or nucleoside analogues. The additional treatment can be either applied prior to, after or concurrently with application of the present invention. The current invention has application to the treatment of virus infections and infections by other intracellular pathogens.

Thus, the present invention provides a process for increasing cellular resistance to a virus of interest. The process comprises two steps. First are provided transformed cells phenotypically resistant to the virus; and a reagent capable of binding to the virus or to a virus-specific site on the cells. Second, the reagent is administered to a biological system containing the cells to increase the resistance of the cells to the virus of interest.

The biological system can comprise an organism, an organ, and a tissue or combinations thereof, viral resistant cells can be eukaryotic or prokaryotic. Such cells can further comprise a nucleic acid sequence selected from

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antisense RNA, antisense DNA, sense RNA, sense DNA, a ribozyme, and a protein binding nucleic acid sequence or combinations thereof.

The virus binding reagent can take various forms including but not limited to an antibody, a virus binding protein, a cell receptor protein and an agent capable of stimulating the production of a virus binding protein or combinations thereof. The antibody can comprise of course a polyclonal or monoclonal antibody which can be specific to an epitope of the virus of interest. The virus binding protein preferably comprises a CD4 receptor; the cell receptor protein preferably comprises a gp24 protein. In addition the production stimulating agent is selectable from an immunological response enhancing adjuvant and a viral antigen or both.

The reagent can be administered in vivo or ex vivo to the cells. Moreover, the process of the instant invention can further comprise administering an additional viral resistance enhancing agent, e.g., a protease inhibitor, a nucleoside analog, or both.

In carrying out the present process the additional viral resistance enhancing agent can be administered before, after, or at about the same time that the binding reagent is administered.

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Also contemplated by this invention are biological systems with increased viral resistance, such resistance having been obtained by any of the processes described above.

9. Dislocation

The present invention is a novel method of altering the concentration of cellular products in a cellular location by the introduction of a construct that produces a product, the dislocation agent, which acts to transport cellular entities from one cellular locale to another. The dislocation agent contains a specificity or affinity domain by which the dislocation agent binds the cellular entity. Dislocation of the cellular entity is mediated by the bound dislocation agent. The resulting co-localization transports the cellular entity to a cellular location that is different from its functional location.

In contrast to previous work (Izant and Sardelli, 1988, Cotten and Birnstiel, 1989, the contents of both publications incorporated herein by reference), which sought to localize the genetic products of their constructs to a cellular location favorable for antisense activity, the present invention acts to disrupt a viral or cellular process by dislocation of macromolecules involved in the viral or cellular processes. Thus, due to the presence of an affinity domain on the dislocation agent, a target molecule will be bound and then transported to a cellular location determined by the dislocation agent.

The application of this invention is through the introduction into cells of nucleic acid constructs which contain sequences for the expression of RNA. The RNA, acting as the dislocation agent, can itself contain sequences for an affinity domain and can transport cellular nucleic acid molecules or proteins to cellular localizations where they are not normally present.

Alternatively, the RNA can bind a target RNA molecule and chaperone it to another cellular location where it can't function by the binding of a protein which transports the RNA dislocation agent and its hybridized target RNA to an unnatural cellular location. Also the RNA can contain a sequence that when translated yields a protein dislocation agent with an affinity domain.

In the current invention, active steps are taken upon the interaction of the target with the dislocation agent. Examples of where this might be useful are RNA molecules that contain signals specifying transport from the cytoplasm into the nucleus. Binding of such an RNA dislocation agent to a cytoplasmic RNA or protein would lead to co-localisation of the target into the nucleus. These transported entities would be unable to function due to their presence in an unnatural cellular location. In a similar way, a protein dislocation agent with an affinity domain for a particular RNA sequence or for another protein can be designed such that it also has a nuclear localisation signal present in its sequence. In this way a target entity, normally present in the cytoplasm, would be localised in the nucleus.

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This invention provides a nucleic acid construct which when introduced into a cell produces a non-natural product. The non natural nucleic acid product comprises two components: a binding component capable of binding to a cellular component; and a localization component capable of dislocating the cellular component when bound to the product. The product from this construct can comprise a protein or a nucleic acid or both. The protein can comprise an antibody, e.g., a polyclonal or monoclonal antibody, such as one directed to a cellular component inside the cell. Such cellular components can comprise any of the following including but not limited to a nucleic acid, a protein, a virus, a phage, a product from another construct, a metabolite and an allosteric compound, or combinations thereof. When comprising a protein the cellular component can comprise a viral or non-viral enzyme, a gene suppressor, a phosphorylated protein, e.g., an oncogene, or combinations thereof.

The binding component of the product produced from the present construct is selectable from a nucleic acid, a protein and a binding entity or combinations thereof. The nucleic acid can comprise a sequence selected from a complementary sequence to the cellular component and a sequence to a nucleic acid binding protein or combinations of both. The protein is selectable from an antibody, a receptor and a nucleic acid binding protein or combinations thereof. The binding entity is capable of binding metabolites.

The localization component is selectable from a nuclear localizing entity, a cytoplasmic localizing entity, and a cell membrane localizing entity or a combination thereof. The localizing component in the present construct can comprise a member selected from a nucleic acid sequence, a nucleic acid structure, e.g., a stem and loop structure and a peptide or oligopeptide, or combinations of the foregoing.

The present invention further provides a process for dislocating a cellular component in a cell. In this process there are provided a nucleic acid construct which when introduced into a cell produces a non-natural product, which product comprises two components. First, there is a binding component capable of binding to a cellular component; and second, a localization component capable of dislocating the cellular component when bound to the product. The nucleic acid construct is introduced into a cell of interest or a biological system containing the cell or cells of interest.

The following is a list or summary of candidate pairs offered for illustration if not by way of limitation. Potential pairs of relocation agents and their targets are presented.

An application of the present invention for the dislocation of cellular macromolecules is the use of a nucleic acid construct that contains a nucleic acid sequence for a U1 snRNA molecule in which a portion of the U1 sequence

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has been substituted with a sequence unique to a portion of the HIV genome (described previously). In this case the U1 RNA in association with snRNP proteins acts as the dislocation agent and the HIV anti-sense sequence represents the affinity domain. The return of U1 to the nucleus, as part of normal cellular processing of U1, while hybridized to target HIV mRNA dislocates the HIV RNA and makes it unavailable for translation in the cytoplasm.

Another application of this invention utilizing U1 RNA is the substitution of HIV packaging signal sequences for a portion of the U1 sequence.

Introduction of the substituted U1 as part of a nucleic acid construct used to transfect cells, provides for the synthesis of a dislocation agent containing the U1 RNA sequences and the HIV packaging sequence signals as the affinity signal. The dislocation agent in this case binds to essential HIV proteins responsible for forming virions and transports them from the cytoplasm to the nucleus, thereby inhibiting the packaging of viral RNA.

Another application of present invention is the use of a nucleic construct which produces an RNA molecule which contains sequences specific for splice junctions of HIV RNA as the affinity domain and sequences for the Rev Responsive Element (RRE) of HIV as an affinity domain for binding to HIV Rev protein molecules which acts as the dislocation agent. In HIV-infected cells, the Rev protein dislocation agent binds to RRE sequences on the RNA

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which is in turn bound to the splice junction of the HIV RNA. The complex would be transported by the Rev protein to the cytoplasm where the unspliced HIV mRNA would be non-functional.

Another application of the present invention is the use of RNA signals for the dislocation of proteins essential for virus replication. The HIV Rev protein is found principally in the nucleolus. However, in the presence of RNA containing RRE sequences, the Rev protein is found principally in the cytoplasm. Therefore, the presence of a nucleic acid construct containing sequences for the cellular production of an RNA dislocation agent containing RRE sequences would actively remove the Rev protein from the nucleus and induce its relocation in the cytoplasm where it would be unavailable for transport of viral RNA. Here the RRE sequences in the transcripts act as the affinity domain.

The many examples which follow are set forth to illustrate various aspects of the present invention, but are not intended to limit in any way the scope of the invention as more particularly set forth in the claims below.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Examples

Example 1 Preparation of a two segment CHENAC in which the Ligands and Chemical Modifications are Localized in One Region of One Segment.

(i) Description of construct

A construct is prepared from one unmodified strand segment and a modified primer segment (Figure 1a). The unmodified single-stranded circle is derived from a plasmid that contains the desired sequences for biological function and it also contains an F1 packaging signal. (Plasmids of this nature are available from a variety of commercial sources.). An E. coli host containing this plasmid is infected with M13 helper phage to obtain single-stranded DNA packaged into phage particles. DNA can then be prepared by a variety of commonly used procedures. The oligomer primer is synthesized with an allylamine phosphoramidite (prepared by the method of Cook et al., 1988) and then modified with tri-lactyl lysyl lysine as described below. The unmodified segment contains a sequence complementary to the modified primer segment. After exposure of the construct to the target cells, the galactose moieties provide binding to their natural receptor and transport the complex into the cell. In the present example, the primer is extended by DNA polymerases in the cell to convert the construct to double-stranded

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form. (Figure 1b) which allows the construct to express sequences specifying biological function in the unmodified region of the CHENAC (designated by the solid black region in Figure 1b). In this example the biological function region of the construct is separated from the region bearing the ligands and chemical modifications.

(ii) Preparation of Lactyl isothiocyanate

p-Nitrophenyl- β -D lactopyranoside (Toronto Research Chemicals, Inc. Catalog # N50385) is converted into p-Isothiocyano- β -D lactopyranoside by the method described by Rafestin et al. (FEBS Letters 40 62-66, 1974).

(iii) Preparation of trilactyl derivative

0.7 g LysylLysine dihydrochloride (Sigma Chemicals) is dissolved in 30 ml of H₂O. 4 g of p-Isothiocyano- β -D lactopyranoside (approximately 8 mMoles) from step (i) is added and the reaction is stirred for 4 hours at room temperature. During this time the pH of the mixture was adjusted to 9.0 and maintained at that value by the addition of 0.2M NaOH. At the end of the reaction, the volume is adjusted to 500 ml with H₂O and loaded onto a DEAE-DE52 cellulose column (previously adjusted to pH 9.0 and then equilibrated with 0.05 M TRIS buffer, pH 9.0). Unreacted LysylLysine remained unabsorbed to the column and is removed by washing the column with 0.01 M LiCl. The product is eluted with 0.1 M LiCl and the fractions from the column are analyzed for UV absorbance at 260 nm. The peak is

collected and the H₂O evaporated under vacuum. The dry residue is triturated with an ethanol/ether (3:1) mixture to remove the LiCl, leaving a solid product. The yield of tri-Lactyl-LysylLysine is approximately 80%.

(iv) Activation of tri-Lactyl-LysylLysine

0.5g of tri-Lactyl-LysylLysine (0.25mMoles), prepared in step (iii), is dissolved in 30 ml of dry Dimethylformamide. 1 g of N-Hydroxy-succinimide is added, followed by 50 mg of Dicyclohexylcarbodiimide. The reaction is allowed to proceed overnight at room temperature. The following day it is evaporated under vacuum. The residue is triturated two times with 25 ml of isopropanol for 30 minutes each at room temperature to remove unreacted Dicyclohexylcarbodiimide and the excess of N-Hydroxysuccinimide. The product is then washed over a filter with absolute ether, the ether removed and the product used without any further purification.

(v) Lactosylation of the nucleic acid portion

1mg of an oligomer designed to be the primer shown in Figure 1 is dissolved in 4 ml of 0.7M LiCl, 0.1 M bicarbonate buffer (pH 7.8). 20 mg of tri-Lactyl-LysylLysine active ester (an approximately 10-fold excess of the reagent compared to the number of allylamine groups) prepared in step (iii) is dissolved in 1ml of Dimethylformamide and added and the mixture was stirred for 6 hours at room temperature. The mixture is evaporated under vacuum and subsequently dissolved in 1 ml of H₂O. The solution is

centrifuged to remove insoluble material and the supernatant was subjected to G50 column chromatography and the DNA fractions combined.

Example 2 A double-stranded version of EXAMPLE 1

The construct described in Figure 1a from EXAMPLE 1 is again used but prior to exposing the DNA to the target cells, the primer is extended *in vitro* by the action of Klenow enzyme (Klenow fragment of DNA polymerase I) to convert the construct into the completely double-stranded DNA molecule shown in Figure 1b. Primer extension is performed under appropriate conditions to avoid strand displacement, for example by carrying out the synthesis at 14°C so that the newly synthesized strand stops at the position of the 5' end of the primer.

Example 3 Preparation of a two segment CHENAC in which one segment has dispersed ligands and chemical modifications.

(i) Description of the construct

A construct is prepared from an unmodified strand segment and a modified primer segment (Figure 2). The modified segment is a DNA oligomer prepared by chemical synthesis such that it contains allylamine deoxyuridine bases as described previously. Peptides are synthesized that contain

sequences for a) a fusogenic peptide derived from influenza (Lear and DeGrado, 1987, J. Biol. Chem. 262: 6500) and b) a peptide promoting localisation to the nucleus of a cell (Kalderone et al., 1984, Cell 39: 499). The peptides are joined to the allyl amine moieties by the procedure given below. The modified primer is complementary to a region in the unmodified segment. The primer is hybridized to the unmodified segment. and extended by Klenow enzyme in the presence of a nucleoside triphosphate mixture containing lactyl-deoxyuridine triphosphate precursors (described below) using the sequence of the unmodified segment as template. Synthesis (polymerization) of the nascent strand is performed at 14°C, so that extension stops at the position of the 5' end of the primer (Figure 2b).

(ii) Synthesis of peptides for addition into the DNA primer

The sequence coding for the Fusogenic Peptide (Gly-Phe-Phe-Gly-Ala-Ile-Ala-Gly-Phe-Leu-Glu-Gly-Gly-Trp-Glu-Gly-Met-Ile-Ala-Gly) and the sequence coding for the Nuclear Localisation Peptide are synthesized chemically with an additional cysteine group added onto the carboxy terminus of each.

(iii) Addition of peptides to allylamines

The allylamine modified nucleic acids are reacted with a 10-fold excess of 3-maleimidopropionic acid N-Hydroxy succinimide ester in 0.7 M LiCl, bicarbonate buffer (pH 7.9) and incubated at room temperature for 40 minutes. At the end of the reaction, the pH is adjusted to 6.0 with acetic

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acid. The unreacted NHS ester (and its hydrolysis product) are removed by extraction with n-butanol two times. The DNA is precipitated with 4 volumes of Ethanol at -70°C . The pellet is then resuspended in 0.1M sodium acetate buffer (pH 6.0) in a minimum concentration of 1 mg/ml. The derivatized DNA is mixed with the desired amount of thiol-containing fusogenic and nuclear localisation peptides from step (ii) and reacted at room temperature for 6 hours. The unreacted maleimido residues on the DNA are quenched by the addition of β -mercapto-ethanol.

iv) Synthesis of Lactyldeoxy UTP

10 μ moles allylamino deoxyUTP (Enzo Biochem, Inc.) are dissolved in 6 ml of 0.7M Lithium Chloride, 0.2M sodium bicarbonate, pH 7.8 and mixed with 20 μ moles of the lactyl-isothiocyanate (described previously) dissolved in 2 ml of Dimethylformamide. The mixture was reacted for 40 minutes at 25°C and then diluted to 100 ml with distilled water and loaded onto a 100 ml bed volume DEAE Sephadex A25 column. The column was washed with 100 ml 0.05 M triethylammonium bicarbonate buffer (pH 7.8) and the product was eluted with a linear gradient of 0.05 M -0.6 M triethylammonium bicarbonate buffer (pH 7.8). The fractions with maximal UV absorbance at 290 nm were collected and the triethylammonium bicarbonate was removed in vacuo in the rotary evaporator at 35°C . The solid residue containing the

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lactyldeoxy UTP is dissolved in 10 mM tris buffer pH 8.0 and used as a substrate for DNA polymerase.

Example 4 Preparation of a two segment CHENAC in which one segment has dispersed ligands and chemical modifications incorporated by ribonucleotide moieties.

A single-stranded DNA construct is derived as described in Example 1 . A second strand made up of RNA is made by incubation of the DNA template with RNA polymerase and a mixture of ribonucleotides according to the method described in Stavrianopoulos et al. (1972, Proc. Nat. Acad. Sci. 69; 2609). Two types of modified ribonucleotides are included in this mixture; lactyl-UTP and allylamine UTP. The allylamine UTP is commercially available (ENZO Biochem, Inc.) and the lactyl-UTP is synthesized as previously described for the lactyl-deoxy-UTP in Example 1 except the ribo derivative of allylamine UTP is used as the starting material. After the RNA strand is synthesized, it is separated from the DNA template strand by melting and then the the allylamine nucleotides were modified further by the addition of fusogenic peptides as described previously in Example 3. The strands were then allowed to reanneal to form the final structure shown in Figure 3.

Example 5 Preparation of a Three Segment CHENAC Containing a Modified Single Stranded Tail.

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(i) Description of the construct

This construct is prepared from two unmodified complementary DNA segments (Segments 1 and 2) and a modified DNA segment (Segment 3). Segment 1 and Segment 2 are hybridized together to form a gapped circle with the gapped region being complementary to Segment 3. The final assembly of these segments are shown in Figure 4. The methods for creating the individual components and assembling them into the final construct are given below

(ii) Preparation of the gapped circle

a) Segment 1 is prepared from plasmid DNA as described previously in Example 1. However, in this particular example, the starting plasmid contains the F(+) packaging signal. Since single-stranded DNA is not a suitable substrate for most restriction enzymes, a small portion of the circular single-stranded DNA is transformed into double-stranded form by hybridization with an oligo that is complementary to an appropriate restriction site. In this example, the restriction enzyme is Sma I and the oligo has been modified by the inclusion of biotinylated nucleotides (Cook, et al. 1988) at the ends. After digestion, the Sma I digested duplex DNA is destabilized and the biotinylated oligo has a much lower affinity. Purification of the cleaved single-stranded linear DNA is achieved by passing the digest over a strepavidin column and collecting the material that does not bind.

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(iii) synthesis of Segment 3

Segment 3 was processed further by the steps given below.

(iv) Addition of modified 3'tail to Segment 3

1 mg of the lactosylated oligomer (Segment 3) is dissolved in 10 ml of a reaction mixture containing 0.2 M cacodylate (pH 6.8), 1 mM deoxythymidine Triphosphate, 0.3 mM allylamine-deoxyuridine triphosphate, 1mM cobalt chloride, 1 mM β -mercaptoethanol and 40,000 units of terminal transferase. The mixture is incubated for 2 hours at 35°C and stopped by the addition of EDTA. Enzyme is removed by absorption to a phosphocellulose column at pH 6.0 and the flow-through is collected, precipitated with ethanol and redissolved in 2ml of 0.1mM EDTA. The final product has a poly-dT tail with approximately 1/4 of the bases containing allylamine groups. Fusogenic peptides are then added onto the allylamine moieties as described previously.

(v) Final assembly

The final construct shown in Figure 4 was formed by the hybridization of the gapped circle created in step (ii-c) with the tailed oligomer created in step (iv) through the complementary of the GAP-1 and GAP-2 sequences.

Example 6 Preparation of a Three Segment CHENAC Containing an Unmodified Single Stranded Tail capable of hybridizing to homopolymers containing Ligands.

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This construct was created in the same manner as the construct described in Example 5, except that after synthesis of the oligomer for Segment 3, the fusogenic peptide was added to the allylamine derivatives instead of the lactyl derivatives and the synthesis of the 3' tail was carried out in the presence of unmodified dATP. As in the previous example, Segment 1, Segment and Segment 3 were assembled together to make a double stranded circle with a 3' single-stranded tail. However, as shown in Figure 6 a further step was added in which segment 4 was added to the complex. This segment was formed by extension of a Thymine tetranucleotide with Terminal transferase in the presence of a mixture of TTP and the lactyl-dUTP in a ratio of 3:1 using the same conditions described previously. Hybridization of Segment 4 to the complex results in the final construct shown in Figure 6.

Example 7 Construction of an RNA derived CHENAC

A construct is made with the appropriate structure shown in Figure 7. Transcription is carried out *in vitro* by use of a T7 promoter directing the synthesis of the sequences of interest. The transcript contains a) sequence A B, which represents a sequence complementary to a lactylated DNA primer (prepared as described previously), b) sequence C D which represents a CMV promoter for directing synthesis of a transcript *in vivo*, c) sequence E F which represents a sequence for biological function which will be expressed after transcription by the CMV promoter and d) sequence G H which is

designed such that its complementary sequence will be a primer binding site similar to the one used by HIV to bind a cellular tRNA^{lys} as a primer for reverse transcriptase. After transcription of the RNA *in vitro*, the modified primer is annealed to the RNA to form the complex shown in Figure 7. This complex could be used either *in vivo*, *ex vivo* or *in vitro* to bind the RNA to a target cell through a ligand/receptor interaction. After endocytosis, some portion of the the RNA should be available in the cytoplasm for further processing and activity. Figure 8 shows the pathway that would occur in the presence of reverse transcriptase activity. This activity can be provided either by targeting a cell that has this activity already present (either intrinsically or due to a retroviral infection) or by introducing it by any of a variety of means known to those skilled in the art. The end result of the steps shown in Figure 8 is a double stranded linear piece of DNA which will be capable of producing transcripts that provide a desirable biological activity.

Example 8 Construction of an RNA derived CHENAC with multiple primers

A construct is made with the appropriate structure shown in Figure 9.

Transcription is carried out *in vitro* by use of a T7 promoter directing the synthesis of the sequences of interest. The construct in this example is similar to the one described in Example 8 except that it is intended to produce an RNA that will be annealed with multiple primers rather than a

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single modified primer. One or more of these primers can be modified. In the present example, the transcript contains a) sequence A B, which represents a sequence complementary to a lactylated DNA primer (prepared as described previously), b) sequence C D, which represents a sequence complementary to a modified DNA primer that has fusogenic peptides attached (prepared as described previously) c) Sequence E F, which is an unmodified primer d) sequence G H which represents a CMV promoter for directing synthesis of a transcript *in vivo*, e) sequence I J K which represents a sequence for biological function which will be expressed after transcription by the CMV promoter and d) sequence L M which is designed such that its complementary sequence will be a primer Binding site similar to the one used by HIV to bind a cellular tRNA^{lys} as a primer for Reverse Transcriptase. For the purposes of clarity, the appended modifications are not depicted in Figure 10. After transcription of the RNA *in vitro*, the primers described above are annealed to the RNA to form the complex shown in Figure 9. This complex could be used either *in vivo*, *ex vivo* or *in vitro* to bind the RNA to a target cell through a ligand/receptor interaction. The ligand modified primer will promote uptake of the complex and after endocytosis the fusogenic peptide modified primer will promote the release of the RNA from the endosomes. Figure 10 shows the pathway that would occur in the presence of Reverse Transcriptase activity. This activity can be provided either by targeting a cell that has this activity already present (either intrinsically or due to a retroviral

infection) or by introducing it by any of a variety of means known to those skilled in the art. The end result of the steps shown in Figure 10 is a series of double stranded linear piece of DNA (each initiated from one of the primers from the complex formed *in vitro*) which will be capable of producing transcripts that provide a desirable biological activity.

Example 9 Construction of a One-Segment Single-Stranded CHENAC.

A construct is made with the appropriate structure shown in Figure 11.

Transcription is carried out *in vitro* by use of a T7 promoter directing the synthesis of the sequences of interest. The transcript contains a) sequence J K, which represents a sequence complementary to a lactyl lysyl lysine modified DNA primer (prepared as described previously) as well as sequences for biological function which include a CMV promoter for directing synthesis of a transcript, a sequence for biological function which will be expressed after transcription by the CMV promoter and a sequence or sequences complimentary to tRNA binding sites. This example differs from the two previous examples in that the complementary DNA is synthesized *in vitro* by using Reverse Transcriptase with the tri lactyl-LysylLysine modified DNA segment as a primer. The resulting RNA/DNA double stranded molecule is treated with Rnase H to yield a single stranded DNA CHENAC.

This complex could be used either *in vivo*, *ex vivo* or *in vitro* to bind the DNA CHENAC to a target cell through a ligand/receptor interaction. After

endocytosis, some portion of the the DNA should be available in the cytoplasm for further processing and activity. Figure 12 shows two possible pathways that could occur after release of DNA into the cytoplasm. Figure 12a shows a pathway similar to that seen in Figure 8 where the construct has been designed such that there is a single tRNA binding site at the 3' end of the DNA CNMAC. Priming and extension *in vivo* by cellular mechanisms result in a single double-stranded DNA molecule. Figure 12b shows a pathway where the construct has been designed such that there are multiple tRNA binding sites at the 3' end of the CNMAC. These can either be identical or different tRNA species can be used. Extension from a CNMAC with sequence for three tRNA primers (as shown in Figure 12b) leads to the synthesis of a double-stranded DNA molecule and two single-stranded DNA molecules. These latter two molecules can be converted into double-stranded molecules if the sequence chosen for the ligand modified primer is also similar to a tRNA primer sequence. When the construct is designed such that the pathway will be similar to that shown in Figure 12a, the construct provide a transcript in which a) sequence J K represents a sequence complementary to the ligand modified primer b) the sequence A B represents a sequence for a CMV promoter c) the sequence C D E F represents a sequence for biological function which will be expressed after transcription by the CMV promoter and d) sequence G H which is designed such that its complementary sequence will be a primer Binding site similar to

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the one used by HIV to bind a cellular tRNA^{lys} as a primer for Reverse Transcriptase. When the construct is designed such that the pathway will be similar to that shown in Figure 12b, the construct provide a transcript in which a) sequence J K represents a sequence complementary to the ligand modified primer b) the sequence A represents a sequence for a CMV promoter c) the sequence B represents a sequence for biological function which will be expressed after transcription by the CMV promoter and d) and sequences C D, E F and G H represent sequences that are complementary to sequence will be primer Binding sites for tRNA's that can be used as primers. The major difference between the net result of the pathways shown in this example and previously described in Example 7 and Example 8 is that the two latter examples depended upon the *in vivo* presence of Reverse Transcriptase whereas the present example provides the Reverse Transcriptase activity *in vitro* prior to binding and uptake into target cells.

Example 10 Preparation of a Double-Stranded CHENAC Containing
Moieties on Each Strand

A construct is made with the appropriate structure shown in Figure 13. Transcription is carried out *in vitro* by use of a T7 promoter directing the synthesis of the sequences of interest. The transcript contains a) sequence A B, which represents a sequence complementary to a lactyl-LysylLysine modified DNA primer (prepared as described previously), b) sequence C D which represents a CMV promoter for directing synthesis of a transcript *in*

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vivo, c) sequence E F which represents a sequence for biological function which will be expressed after transcription by the CMV promoter and d) sequence G H which is identical to the sequence of a second modified primer that has fusogenic peptides attached (prepared as described previously). In Figure 10, the lactyl ligands are depicted by X X on the first primer and the fusogenic peptides are shown as Z Z in the second primer. DNA is synthesized *in vitro* by using the transcript as a template for Reverse Transcriptase with the tri lactyl lysyl lysine modified DNA segment as a primer. The resulting RNA/DNA double stranded molecule is treated with Rnase H to yield single stranded DNA. The second primer containing the fusogenic peptides is then used as a primer to prepare the complimentary second strand of DNA.

This complex could be used either *in vivo*, *ex vivo* or *in vitro* to bind the DNA to a target cell through a ligand/receptor interaction. The ligand modified primer will promote uptake of the complex and after endocytosis the fusogenic peptide modified primer will promote the release of the DNA from the endosomes.

Example 11. A Bifunctional Binder Composed of a Bispecific Antibody

The methods of recombinant DNA are used to prepare a bispecific antibody with specificities for the CD4 protein of lymphocytes and for murine leukemia

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virus (Figure 14). The antibody is prepared from murine monoclonal antibodies according to the procedure of Staerz and Bevan (1985 Proc Natl Acad Sci USA **83**;1453) for the production of hybrid hybridomas..

Antibody modifications. Hydrazine groups are introduced to antibodies in the carbohydrate moieties after oxidation with periodate or galactose oxidase and subsequent reaction with hydrazine. When galactose oxidase is used for antibody oxidation, it is necessary to analyze for free galactose groups as follows. The antibody is oxidized with galactose oxidase in the presence of a peroxidase. At the end of the reaction the mixture is reacted with Lucifer Yellow CH (Aldrich) and passed through a G50 column. If the flowthrough from the column fluoresces, this is an indication that the antibody contains free galactose residue and that the galactose oxidase can be used for antibody activation.

Ten mg antibody are dissolved in 1 ml of 0.1M acetate buffer, pH 5.0, and oxidized with 1.0 umole NaIO_4 at 4°C for 30 minutes. Excess periodate is removed by Sephadex G50 (Pharmacia) chromatography in 0.05M acetate buffer, pH 5.0. The protein fractions are combined and reacted with 1.0 umole hydrazine acetate, pH 5.0, for 30 minutes at room temperature. The pH is raised to 9.0 with sodium carbonate and the contents are cooled to 0° and 10 umoles sodium borohydrate are added in three portions at ten minute intervals. The reduction is continued for an additional 60 minutes and the

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Oligonucleotides synthesized with a thiol group at the 5' end or the thiol groups were added by reaction with an allylamine residue at the 5' or 3' end of the nucleic acid with homocysteine thiolactone at pH 9.0.

A single stranded DNA molecule 120 bases in length and containing a 5' terminal nucleotide modified by the addition of an allylamine group is

prepared chemically by the method of Cook et al. (1988 Nucleic Acids Res 16;4077). and the allylamine residue is thiolated as in Example 11. The 70 bases at the 3' end are complementary to the single stranded region of Adeno Associate Virus DNA. The single stranded DNA is attached to the F(ab')₂ fragment as in Example 11 and they anneal to Adeno Associated Virus as indicated in Figure 15.

Example 13. A Binder Composed of a Bispecific Antibody (or of the F(ab')₂ Fragment of a Bispecific Antibody) Attached to a Single Stranded DNA Domain for the Nucleic Acid Component (Figure 16).

A bispecific antibody is prepared as described in Example 11 from a murine monoclonal antibody to CD34 cell surface protein and and a murine monoclonal antibody to adenovirus. The single stranded DNA molecule described in Example 12 is attached to the bispecific antibody (or to the F(ab')₂ fragment of the bispecific antibody) and annealed to the adeno associated virus. An inactivated adenovirus is bound to the antibody (Cristiano et al. 1993 Proc Natl Acad Sci USA 90;2122: Curiel et al. 1991 Proc Natl Acad Sci USA 88;8850) in order to facilitate cellular uptake of the complex.

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Example 14. A Binder composed of a Domain for Adeno Associated Virus DNA, a Domain for Binding to Liver Cells and an Inactivated Adenovirus (Figure 17).

Preparation of lactyl oligolysine 10mer. Oligolysine is synthesized containing a cysteine residue at the carboxy terminus. The thiol group is blocked with Ellman's reagent and the amino groups are reacted with a threefold excess of lactylisothiocyanate in 0.1M bicarbonate buffer, pH 9.0, and 20% dimethylformamide for 2 hr at room temperature. The reaction mixture is chromatographed on a G50 column and the lactyl-oligolysine fractions are combined and freeze dried. The solid is dissolved in 2 ml 1mM dithiothreitol to unblock the protected thiol group and chromatographed again on a G50 column to remove the excess dithiothreitol and the liberated Ellman's reagent. All operations are performed with argon saturated buffer to prevent thiol oxidation by air. The combined lactyl oligolysine fractions are combined and reacted immediately with the maleimide derivatized antibody (see below) or proteins in a mixture with thiol containing nucleic acid as in Example 12.

Example 15. An Antibody Binder with an Attached DNA with Domains for Adeno Associated Virus DNA and for Binding to Liver Cells (Figure 18).

A single stranded DNA molecule 100 bases in length and with a 5' terminal nucleotide containing a thiol group is synthesized chemically (Allylamine groups are interspersed at 10 base intervals along the 50 bases at the 5' end of the molecule Cook et al.) and the 50 bases at the 3' end of the molecule are homologous to adenovirus associated virus DNA. After blocking the thiol groups, the lactyl groups are added as described in Example 11. The thiol groups are then unmasked and the lactyl modified single stranded DNA is added to a murine monoclonal antibody to adenovirus and it is annealed to adenovirus associated virus DNA as described in Example 12.

Example 16 Preparation of a multimeric Antibody by means of
Nucleic Acid Hybridization

(i) Preparation of homopolymer

Oligo(dA) and oligo(dT) with an amine group at the 5' end were synthesized chemically. Longer molecules were prepared by using the amine-containing oligos as primers in a reaction with Terminal transferase and the appropriate dNTP precursors depicted as NA in Figure 19 and 20..

(ii) Preparation of homopolymer linker

1,2 Diamino-4-Bromo-5-Hydroxycyclohexane was prepared according to U.S. Patent No. 4,707,440 where the product of the (11-5) reaction was reacted with N-Bromosuccinimide as in step (4-7) to yield compound I. (The various steps in this synthesis are shown in Figures 19 and 20). Compound I was

reacted with a 5-fold excess of dithiothreitol at 90°C, pH 8.0 in argon atmosphere for 2 hours. The reaction mixture was acidified to pH 1.0 and the excess of dithiothreitol was removed by peroxide-free ether until no thiol was detected in the ether phase. The aqueous phase which contains Compound II was used for the next step.

(iii) Attachment of linker to homopolymer

The 5' amino group of the nucleic acid was reacted with 3-maleimidopropionic acid N-hydroxy succinimide ester in 0.2 M sodium bicarbonate buffer pH 7.8 and 0.7 M lithium chloride 30% dimethyl formamide for 40 minutes at 25°C. The pH of the mixture was brought to 5.5 with 2.0 M acetic acid and the excess active ester was removed by extraction with n-butanol. The product Compound III was precipitated with 4 volumes ethanol for 2 hours at -70°C. It was centrifuged and the pellet was dissolved in 0.7 M lithium chloride and reacted immediately with excess Compound II at pH 6.0 for 30 minutes at room temperature to yield Compound IV; it was separated from excess of Compound II by ethanol precipitation as in the previous step. Compound IV was reacted with excess 3-maleimidopropionic acid N-hydroxy succinimide ester (as described in the preparation of Compound III) to yield Compound V. The product was precipitated twice with 4 volumes ethanol and stored as a pellet at -70°C until used.

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(iv) Preparation of Antibody

Fab'-SH fragments were prepared by reduction of F(ab')₂ antibody with 0.5 M dithiothreitol at pH 7.5 (Taizo Nitta, Hideo Yagita, Takachika Azuma, Kiyoshi Sato and Ko Okumura Eur J. Immunol 1989 19: 1437-1441) under argon atmosphere. The pH was lowered to 6.0 and the antibody was separated from dithiothreitol by G50 chromatography using fully deaerated buffer under argon atmosphere to prevent oxidation to F(ab')₂

(v) Attachment of homopolymer to Antibody fragments

The protein fractions from step (iv) were combined and reacted with Compound V (Figure 20) from step (iii) in a 2:1 ratio to form Compound VI, always under argon atmosphere and in the presence of 2mM EDTA to prevent nuclease action. After overnight incubation at 4°C, Ethylmaleimide was added to the reaction mixture to block any free thiol residues and the protein was precipitated with ammonium sulfate (60% of saturation). The pellet was dissolved in minimum amount tris-HCl buffer, pH 7.8 and chromatographed in a G100 column to separate the conjugate from the reaction products.

(vi) Annealing of homopolymers to obtain Antibody multimers

Annealing is done 0.2M NaCl, .05M Tris·HCl (pH 7.8), 1mM EDTA. Figure 21 shows the overall outline of the process. In the last step shown in figure 21, (a) shows an example where both the A homopolymer and the T homopolymer are short enough that there is essentially only one of each type of molecule binding together in a 1:1 ratio. The (b) diagram shows the situation where the A homopolymer was synthesized such that its much longer than the T homopolymer; in this situation, larger numbers of antibodies can be linked together into complexes.

Example 17 Preparation of a multimeric insulin by means of
Nucleic Acid Hybridization

Oligo T with a primary amino group (prepared as described earlier) is reacted in 0.7M LiCl , 0.1M sodium bicarbonate buffer, pH 7.8 and 30% dimethyl formamide with a 3-fold excess of suberic acid bis (N-hydroxysuccinimide) ester for 15 minutes at room temperature. The pH was then lowered to 5.0 by the addition of 2M acetic acid and the excess of active ester was extracted twice with n-butanol. The nucleic acid was precipitated with 4 volumes ethanol at -70°C and the pellet after centrifugation was dissolved in cold 0.7 M LiCl in 0.1 M sodium bicarbonate solution (pH 7.8), solid insulin was added in 1:1.2 ratio and the conjugation was allowed to take place at 4°C overnight. The product is separated from the reactants by molecular

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sieving chromatography on G75 columns. A multimeric complex is formed by the hybridization of the T-tailed insulin molecules with a Poly A binder as described earlier. The steps in this Example are shown in Figure 22.

Example 18 Preparation of a multimeric insulin by means of
Nucleic Acid Hybridization through specific discrete
sequences.

A group of nucleic acid sequences are selected from the known sequence of the single-stranded form of bacteriophage M13. These are then artificially synthesized such that they have a primary amino group on the nucleotide at the 5' end. the oligomers are individually activated and attached to insulin molecules as described in Example 17. A mixture is made of each of the oligomer/insulin complexes and mixed with M13 DNA derived from phage particles (the + strand). The product was separated from the reactants by molecula sieving chromatography. The steps in this Example are shown in Figure 23.

Example 19 Synthesis of a Eukaryotic Vector that Expresses T7 RNA
Polymerase as well as Antisense Sequences Directed by a T7
Promoter.

(A) Intron and Intron Insertion Site

The SV40 small T intron has been utilised in a number of DNA vectors and it has been chosen for this particular example due to its small size and the presence of stop codons in all three reading frames. The consensus sequences for splice donors and acceptors are partially made up by exon sequences as well as intron sequences. A computer search using the MacDNASIS program (Hitachi, Inc.) allowed the identification of 19 different sites within the T7 RNA polymerase coding sequence (Mount, 1982 Nucleic Acids Research **10**,459) that contain the sequence (C/A)AGG, which as described earlier is a consensus sequence for a post-splice junction. Any of these sites should be suitable for the intron insertion site, but for this example, a T7 site was chosen that closely resembled some of the flanking exon sequences of the SV40 intron. Figure 24 shows the sequences surrounding this site in the T7 RNA polymerase gene sequence and the subsequent insertion of the SV40 virus intron into this site. Figure 24 also shows the mRNA made from this fusion and the subsequent splicing out of the Intron sequence to reconstitute the normal T7 coding sequence.

(B) Fusion of intron sequences into the T7 coding sequences

A method for introduction of the intron and production of a vector that contains the interrupted T7 RNA Polymerase as well as sequences directed from a T7 promoter is given in Figure 25. As shown in Figure 25, the

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creation of this construct can be accomplished by PCR amplifications of each segment of the T7 RNA polymerase gene (left and right of the intended intron insertion site) and PCR amplification of a eucaryotic intron. These pieces are joined together using cloning steps described below. It has previously been shown that PCR products can be fused together by a technique referred to as "Splicing by Overlap Extension" (SOE) to generate precisely joined fragments without extra sequences being added (Horton *et al*/ 1990 BioTechniques 8: 528; Horton *et al.*, 1989 Gene 77: 61). However, in addition to the PCR reactions needed to create the different segments, the SOE method involves the use of these PCR products as primers in a secondary PCR reaction to fuse the segments. For fusions of multiple segments there would be a series of sequential PCR reactions to be carried out. Even with thermostable DNA polymerases chosen for a lower error frequency, the synthesis of the final product will require that some sequences be subject to several multicycle amplification steps thereby leading to an increased chance of undesirable mutations in the final product. For this reason, the inventors of the technique advised sequencing the final product to insure that the desired product was obtained (Horton *et al.*, 1990). In the present example, a method was used that requires only an initial round of PCR amplification to create each segment followed by ligation of the segments together to form the final fused product. Fusions of the gene segments and intron to form the appropriate product were carried out

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by addition of restriction enzyme sequences onto the 5' end of the PCR primers to allow the production of "sticky ends" (Scharf *et al.*, 1986 Science 233: 1076). To give the precisely defined end points for this fusion, restriction enzymes (Bsa I and Bsm B1) that recognize non-palindromic sequences and cut outside of their recognition sequence to leave a single stranded tail with arbitrary definition were used. This method allows joining of sequences at any point chosen by the user by the appropriate design of the PCR primers.

(C) Synthesis of the individual segments used for the fusion.

The T7 RNA polymerase is encoded by bases 3171-5822 in the T7 genome (Dunn and Studier, 1983 J. Mol. Biol. 166: 477) and this sequence is available in Genbank as Accession #'s V01146, J02518 or X00411. Based upon this information, six different oligos were synthesized. The use of these oligo's and their sequences are given in Figure 26. TSP 1 and TSP 2 were annealed together by a 12 bp complimentary sequence and extended to form a completely double-stranded DNA molecule (Figure 27). Conditions were as follows: 150 pM of TSP 1, 150 pM of TSP2, 1X NEB Buffer #2 (New England Biolabs, Inc.), 200 uM dNTP and 13 units of Sequenase v2.0 (U.S. Biochemicals, Inc) for 75 minutes at 37° C. TSP 3 and 4 were used in a PCR reaction (Saiki et al. 1985 Science 230, 1350)) with T7 genomic DNA as a template to synthesize the "Left" fragment. Reagent conditions were as follows: 100 ul volume containing 100 ng T7 template (Sigma

Chemical Co.), 1 uM TSP 3, 1 uM TSP 4, 1mM MgCl₂, 1x PCR buffer, 250uM dNTP, 2.5 units of Taq DNA Polymerase. Temperature cycling conditions were: 16 cycles of (1) 50 seconds at 94° C (2) 25 seconds at 50° C and (3) 3 minutes at 72° C. The same conditions were used to form the "Right" end fragment with Oligomers TSP-5 and TSP-6 except that due to the length (over 2 kb) of the expected product, 2.5 units of Taq Extender (Stratagene, Inc) was added and the Taq Extender buffer substituted for the normal PCR buffer. INT-1 and INT-2 were used together in a PCR reaction to form the Intron piece. Conditions were the same as those used for synthesizing the "Left" fragment of T7, except that a clone of SV40 was used as the template and due to the smaller size of the amplicon, the cycle conditions were only 1' at 72° C for the extension time. Figure 27 shows the synthesis of the short double stranded piece of DNA made by extension of oligo's TSP 1 and TSP 2 and its combination with the left end of the TSP 3/TSP 4 PCR product to generate the complete (NLS +) T7 RNA polymerase. The resultant nucleic and amino acid sequences are given in Figure 28 for the construct given in this example as well as the normal wild type T7 RNA polymerase sequences.

Thus, the modifications carried out at the 5' end during this construction process were:

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a) The sequence around the ATG start codon was changed to give a Kozak consensus sequence (Kozak 1984 Cell 44: 283) to increase efficiency of translation of the gene product. This change had previously been introduced into the T7 RNA polymerase coding sequence.

b) The fusion of the TSP1/TSP2 extension product to the TSP3/TSP4 PCR introduces a 9 amino acid insertion between bases 10 and 11 in the normal T7 RNA polymerase protein sequence. This sequence has previously been shown to be a signal for transportation to the nucleus by Kalderone et al. (1984 Cell 39: 499) and had been introduced into T7 RNA polymerase by Lieber et al., (1989) as a substitute for the first 10 amino acids and inserted into an artificially created EcoR1 site by Dunn et al., (1988). The method used in this Example to introduce the Nuclear Localisation Signal (NLS) was designed to minimize perturbations to the normal structure of the protein. The codons for the amino acids coding for the NLS are indicated as larger type size in Figure 28

(D) Combination of pieces to form the final construct of the T7 RNA polymerase gene in a eucaryotic expression vector

Figure 29 shows the various steps used for this process. For ease of use, each of the three pieces (PCR #1, PCR #2 and PCR #3) was cloned into a

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plasmid vector (PCR II) using the TA cloning kit and following the manufacturer's instructions (Invitrogen, Inc.).

PCR #1 (the left end of the T7 RNA polymerase) was cloned into PCR II to create pL-1. This construct was then digested with BsmB1 and Spe I to excise out the PCR product and the TSP1/TSP2 Extension product (shown in detail in Figure 27) was digested with Eco R1 and Bsa I. Due to the design of the primers, the single-stranded tails created by BsmB1 and Bsa I are complimentary to each other and ligation of these pieces forms a single piece with an EcoR1 tail at one end and a Spe I tail at the other end. Digestion of the M13 vector, mp18, with EcoR1 and Xba I allows insertion of the EcoR1/Spe I piece to form pL-2.

PCR #2 (the SV40 Intron) was cloned into PCR II to form pINT-1. This construct was digested with EcoR1 and Spe I and transferred into the M13 vector (mp18 digested with EcoR1 and Xba I) to form pINT-2.

PCR #3 (the right end of the T7 RNA polymerase) was cloned into PCR II to create pR-1. This construct was digested with Eco R1 and Spe I and then self-ligated to form pR-2. This step was added to eliminate extra EcoR1 and Spe I sites present in pR-1.

As described in Figure 25, the elements in pL-2, pINT-2 and pR-2 are fused together to form the complete intron-containing T7 RNA polymerase. This was accomplished by digestion of pL-2 with BsmB1 and Bsa I; pINT-2 with BsmB1; and pR-2 with BsaI and Spe I. Ligation of these three inserts together forms a single fragment that has one end compatible with a Hind III end and the other end compatible with Spe I. This fragment was cloned in the same step into pRc/RSV (from Invitrogen, Inc.) that had been previously digested with Hind III and Spe I. As shown in Figure 29, this final product is pINT-3. This particular eucaryotic vector was chosen since it had been shown previously that the RSV promoter is especially active in hematopoietic cell lines. Also, the ligation of the Hind III end from pRcRSV to the end created from the BsmB1 digestion of pL-2, does not reconstitute the Hind III site in pINT-3, the final product.

E) Antisense sequences

Three different targets in the HIV genome were chosen as test targets for Antisense: (A) the 5' common leader, (B) the coding sequence for Tat/Rev and (C) the splice acceptor site for Tat/Rev. Antisense to (A) was derived from a paper by Joshi et al. (1991 J. Virol. **65**,5534); Antisense to (B) was taken from Szakiel et al. (1990 Biochem Biophys Res Comm **169**, 213) and the Antisense to (C) was designed by us. The sequences of the oligo's and their locations in the HIV genome are given in Figure 30. Each oligo was designed such that annealing of a pair of oligo's gives a double-stranded

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molecule with "sticky ends" that are compatible with a Bam H1 site. The oligo's were also designed such that after insertion into a Bam H1 site, only one end of the molecule would regenerate the Bam H1 site, thus orientation of the molecule could easily be ascertained. The resultant clones were termed pTS-A, pTS-B and pTS-C for the anti-HIV sequences A, B and C respectively.

F) Cloning of T7 terminator

The sequence for termination of transcription by the T7 RNA polymerase is encoded by a sequence between the end of the gene 10b protein at base number 24,159 and the start codon of the gene 11 product at base number 24,227 in the T7 genome (Dunn and Studier 1983 J. Mol. Biol. **166**, 477) Genbank Accession #'s V01146, J02518 or X00411. Based upon this information, TER-1 and TER-2 were synthesized (Sequences given in Figure 30) and used in a PCR amplification reaction to obtain a double-stranded 138 bp piece that contained the T7 sequences from 24,108 to 24,228 with an Xba I site added at one end and a Pst I site added to the other. The reagent conditions for amplification were as described for the TSP3/TSP4 reaction but the temperature cycling conditions were: 16 cycles of (1) 50 seconds at 94° C (2) 25 seconds at 50° C and (3) 1 minute at 72° C. As shown in Figure 30, the terminator piece was cloned into the PCR II vector and then after XbaI/Pst I digestion it was transferred into an M13 vector.

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G) Creation of T7 driven antisense transcription units.

The clones containing Antisense sequences (pTS-A, pTS-B and pTS-C) were digested with Eco R1 and Pst I while the clone containing the T7 terminator (pTER-2) was digested with Xba and Pst I. These were ligated together with pBI 30 (IBI, Inc.) that had been digested with Eco R1 and Pst I to form the AntiSense transcription units shown in Figure 30 which have Antisense sequences transcribed from a T7 promoter and then terminated by a T7 terminator. The resultant clones were termed pTS-A1, pTS-B1 and pTS-C1 for the anti-HIV sequences A, B and C respectively.

H) Transfer of Antisense Transcription Units into pINT-3

By the nature of the present invention, the T7 driven Antisense Transcriptions units can be transferred into pINT-3 to make a single construct T7 polymerase/promoter construct. This was accomplished by creating an M13 phage vector LIT Ø-2 by transferring the polylinker from the plasmid vector LIT-38 (New England Biolabs, Inc.) by digestion with Spe I and Sph I and ligating the polylinker insert into mp18 that had been digested with Xba I and Sph I. This and subsequent steps are shown in Figure 31. Clones pTS-A1, pTS-B1 and pTS-C1 which contain T7 directed Antisense sequences were digested with EcoRV and Pst I. They were then ligated to the LIT Ø-2 vector which had also been digested with Eco RV and PstI. The resultant clones are phage vectors that contain T7 directed Antisense sequences and

were termed pTS-A2, pTS-B2 and pTS-C2 respectfully. These clones were digested with Nhe I and Bsp 120 I and ligated to the pINT-3 vector (from Figure 29) that had previously been digested with Spe I and Not I. the resultant clones pRT-A, pRT-B and pRT-C contain the coding sequence for the T7 RNA polymerase driven by the RSV promoter and with an SV40 intron sequence that will be spliced out to form a functional polymerase enzyme and in addition each construct contains an HIV Antisense sequence driven by a T7 promoter and terminated by a T7 terminator.

Example 20 Expression of a protein made from T7 directed transcripts
derived from a single construct that also expresses the T7 RNA
polymerase

The pINT-3 vector used in the previous example can be modified for use as an expression vector for T7 directed protein synthesis. For this purpose, the pINT-3 vector needs has a T7 promoter, a T7 terminator and a polylinker in between. The optimal site for the placement of these moities is after the poly A signal for the T7 RNA polymerase in pINT-3 where there is an Xho I and a Bam H1 site. Since there are also other Xho I and Bam H1 sites within the vector, manipulations of this particular segment can only be done if the small segment containing this area is separated out, the appropriate nucleic acids introduced in between the Xho I and Bam H 1 sites and then the segment replaced back in. The steps used for the creation of this construct are shown in Figures 32 and 33.

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a) Introduction of polylinker

The segment containing the Xho/Bam H1 insertion site was derived from the plasmid pRC/RSV, which was the parent of pINT-3. This was done by digesting pRC/RSV with Xba I and Xma I and transferring the appropriate fragment into the plasmid pUC18 (NewEngland Biolabs, Inc.) previously digested with Xba I and Xma I to obtain the vector pEXP-1. This in turn was digested with Xho I and Bam H1 and then a polylinker was inserted by ligation with oligomers PL-1 and PL-2 (Sequences are shown in Figure 32). The resultant plasmid was named pEXP-2 and the restriction sites contained with the new polylinker are shown in Figure 32.

b) Introduction of T7 promoter and T7 terminator

A promoter was inserted into pEXP-2 by digestion with Nco I and Bam H1 followed by ligation with oligomers TPR-1 and TPR-2 (Sequences are shown in Figure B-10) to create pEXP-3. The normal T7 promoter consensus sequence (Dunn and Studier, 1983) was not used since it has been shown that it can function as a eucaryotic promoter in some cell lines (Sandig et al., 1993 Gene 131;255) and a sequence derived from Lieber et al. (1993) was substituted since this equence still functions well in the presence of T7 RNA Polymerase but remains silent in its absence. The vector pEXP-3 was digested with Spe I and Pst and ligated to the T7 terminator fragment derived from the pTER-1 construct described in the previous example in order

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to create the vector pEXP-4. The Xba/Xma segment has now been modified to contain the T7 terminator, a short polylinker and the T7 terminator. It was substituted for the unmodified segment in pINT-3 by Xba I/ Xma I digestion of pINT-3 and PEXP-4 followed by ligation as shown in Figure 33 thus creating the vector pINT-4.

c) Introduction of a protein coding sequence into the new T7 Expression vector

The gene coding for the complete lac Z sequence was obtained from pZeoSVLacZ (Invitrogen, Inc.) by digestion with Age I and Cla I. This was then ligated into pINT-4 that had been previously digested with Bsp E1 and ClaI to create pINT-LacZ (not shown). After introduction into a eucaryotic cell, the RSV promoter directs the synthesis of the T7 RNA polymerase which in turn acts upon the T7 promoter to synthesize B-galactosidase.

Example 21. A Primary Nucleic Acid Construct that Propagates Production
Centers for the Production of Produces Single-Stranded
Antisense

A Primary Nucleic Acid Construct is described as shown in Figure 34 and 35 whereby, subsequent to introduction into a cell, a series of events, including self priming, multiple priming and Rnase H and reverse transcriptase

activities, leads to the production of single stranded DNA antisense molecules. In this case a Nucleic Acid Construct creates multiple copies of a Production Center, an RNA transcript with hairpin structure with a discrete 3' end (structure 34a, Figure 34). In the presence of reverse transcriptase self priming occurs by the 3' end of the hairpin acting as primer to extend to the 5' end of the molecule resulting in a hairpin structure composed of both DNA and RNA (structure 34b). By a multiple priming process, RNase H, either as part of the viral reverse transcriptase or from the Inherent Cellular Systems, starts degradation of the RNA bound to the DNA. Degradation can be complete if there is enough RNase H activity, or if the reverse transcriptase activity is high enough, the initiation of RNA degradation provides RNA fragments that serve as primers for extension using the DNA portion as a template. In the former case the net result of the degradation by RNase H is a single-stranded DNA molecule with a double stranded 5' RNA terminus (structure 34c); in the latter case (structure 34d), the priming event results in a) the Production of a series of molecules such as 34f and 34g, the length of the single-stranded DNA portion depending upon the site of the priming initiation event and b) the propagation of Production Centers such as structure 34e. Structure 34g could act as a biological modifier if, for example, the sequences represented as the Z single stranded DNA region were antisense sequences. Through the activity of RNase H and reverse transcriptase, structure 34e would be processed further to produce single

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stranded DNA molecules (structures 351h, 35i and 35j, Figure 35). which could act as antisense DNA if the sequences X', Y', Z' were designed with that purpose. The Production of antisense DNA molecules according to this invention represents the first demonstration of the method for the intracellular synthesis of antisense DNA.

Example 22 A Primary Nucleic Acid Construct that Propagates an RNA Production Center that is Reverse Transcribed to Create DNA Production Centers Capable of Directing Transcription.

In this example, the same processes of self priming and multiple priming described in the Example 21 occur with the propagation of single stranded DNA hairpin structures (Figure 36). As in Example 21, structures structures 36b, 36c and 36d (Figure 36) act as Production Centers for the Production of single stranded RNA. In this case this represents an amplification event since reverse transcriptase and RnaseH convert a single Production Center (36a), into a double stranded DNA Production Centers (36b, 36c and 36d) which can direct the Production of multiple single stranded RNA molecules.

Example 23 A Primary Nucleic Acid Construct which Propagates a Double Hairpin Production Center for the Production of Single Stranded RNA

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26 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100

In this example, a double stranded DNA Primary Nucleic Acid Construct (structure 37a, Figure 37) has been designed such that a single stranded Production Center, propagated from it, forms hairpin structures at the 5' and 3' ends. Extension by self priming from the 3' end followed by further steps catalyzed by RnaseH and reverse transcriptase result in the propagation of a double-stranded DNA molecule with single stranded hairpin ends (structure 38b, Figure 38). This can be further processed, by the action of DNA ligase, to form a covalently closed molecule (38c) or by the action of reverse transcriptase to form a larger linear molecule (38d). The presence of promoters and coding sequences in these Production Centers provides for Production of single stranded RNA. As seen above in Example 22, this is an amplification event since each Production Center producing RNA transcripts was itself derived from a single transcript.

Example 24 A Nucleic Acid Construct which Propagates a Production Center capable of Inducible Cell Destruction.

In this example (Figure 39) provides for the production a single stranded nucleic acid as a result of the introduction into cell of an inherent cellular system. In this case, the events leading to the Propagation of a Production Center (structure 39b) are brought about by the presence of Reverse Transcriptase. Here, the single stranded nucleic acid product of a Production Center is mRNA which can be translated to produce a lethal product,

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diphtheria toxin, resulting in a reverse transcriptase dependent cytotoxic event. Elimination of low level synthesis of a toxic gene product such as diphtheria toxin in the absence of viral infection TAT activation (as was observed by Harrison et al.) is accomplished by the use of an intron artificially inserted into the non-coding strand (39a) of the segment coding for the toxin. In this way, transcription of the toxin sequence will not produce an active product. Production of active toxin only occurs when the antisense transcript is spliced and used as a template for Reverse Transcriptase.

The result of RNase H and reverse transcriptase mediated activities is a double stranded DNA Production Center (39c) that has a template for the toxin and which has the intron sequences removed. As a further refinement, the promoter sequence in the double-stranded DNA Production Center (region designated as ABC in structure 39b) can be an HIV LTR. In this case Production of the toxin would be dependent upon two events that should be provided by viral infection.

Example 25 Use of tRNA Primers to Create a Double-Stranded DNA

Production Center for Production of Single Stranded RNA.

This example utilizes the presence of primer binding sites in a single stranded RNA Production Center for the Propagation of a double-stranded DNA Production Center. In this way, sequences derived from the Primer Binding Sites of retroviruses, such as the HIV primer binding site which utilizes lysyl

tRNA as a primer, can be inserted near the termini (regions designated X and Y) in the RNA Production Center (Figure 40, structures 40b and 40c) for the priming of DNA synthesis to form double stranded DNA Production Centers. The resultant Production Centers, such as structure 40d, are double stranded DNA molecules but can function as described previously to produce single stranded RNA which either can be utilized as anti-sense nucleic acid or which can be translated to produce a protein.

Example 26 Construction of plasmids with Anti-sense segments introduced into the transcript region of the U1 gene.

The overall process used in this example is depicted in Figure 41. The gene for U1 is present in the plasmid pHSD-4 (Manser and Gesteland 1982 Cell 29;257). Three different pairs of deoxyoligonucleotides were synthesized and the sequences are given in Figure 42. The pairs were hybridized to form double stranded molecules with single stranded overhangs to form sites compatible with the Bcl/Bsp ends in the plasmid. The Bcl/Bsp ends in the plasmid remain after removal of the 49 base sequence from the U1 coding sequence. When each sequence is inserted into and expressed from the U1 coding region of pHSD-4 U1 it will appear as an antisense RNA sequence to a region of the HIV genome.

After digestion with Bcl 1 and Bsp E1, a 49 base pair segment is eliminated from the U1 transcript portion of the gene. The oligo pairs have been

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designed to form sticky ends compatible with the Bcl/Bsp ends in the plasmid. Ligation of each of the pairs of Oligo's (HVA-1 + HVA-2, HVB-1 + HVB-2 and HVC-1 + HVC-2) created pDU1-A with an insertion of 72 bp, pDU1-B with an inserion of 66 bp and pDU1-C with an insertion of 65 bp. As a control, two oligomers (HVD-1 and HVD-2) with sequences unrelated to HIV were also inserted into the U1 operon to create pDU1 which contains an insertion of 61bp.

To allow for selection of transformants after introduction of these chimeric U1 genes, the Neomycin resistance gene was introduced by digestion of pGK-neo (McBurney et al. 1991 Nucleic Acids Research 19;5755) with Hind III and Sma I and ligation into the pDUI series of plasmids previously digested with Hind III and Hinc II to create the pNDU1 series (pNDU1-A, pNDU1-B, pNDU1-C and pNU1-D).

As described earlier, the design of the cloning method should allow the insertion of novel sequences that would still allow the utilisation of signals provided by the U1 transcript for nuclear localisation of Anti-sense sequences. To test whether the insertion of the sequences described above resulted in unintended changes in the U1 region responsible for re-importation of the U1 transcripts a computer analysis was done to compare the predicted structures for the normal U1 and the chimeric novel molecules using the MacDNASIS program (Hitachi, Inc.). In Figure 43 it can be seen

that despite changes in the 5' end (where the new sequences have been introduced) loops III and IV as well as the Sm region remain undisturbed.

Example 27 Construction of a Multi-Cassette Construct which Expresses
Three Antisense Sequences as Part of U1 snRNA

The various steps used in this example are depicted in Figure 44. The various constructs used in this example, pDU1(A), pDU1(B), pDU1(C) and pGK-neo were described in Example 26 of this patent. The plasmid pDU1(B) with the "B" anti-sense embedded within the U1 transcript was digested with Sma I and Hind III. The segment containing the U1 operon with the "A" anti-sense was released by digestion of pDU1(A) with Hinc II and Hind III and ligated into the pDU1 (B) plasmid to create pDU1(A,B) which contains two separate operons for the "A" and "B" anti-sense sequences. This construct was then digested with Sma I and Hind III (to release the double operon) and ligated into pDU1(C), containing the U1 operon with the "C" anti-sense, that had previously been digested with Hinc II and Hind III. The resultant construct, pDU1(A,B,C) contains three separate operons containing the "A", "B" and "C" anti-sense sequences. To allow selection for the presence of this construct after a transfection step, the segment containing Neomycin resistance was excised from the vector pGK-neo by digestion with Hind III and Sma I and ligated into the pDU1(A,B,C) construct to create pNDU1(A,B,C). The ordering of the three operons in the pDU1(A,B,C) and pNDU1(A,B,C) constructs is given in Figure 46.

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Example 28 Construction of an Antisense Expressing Multi-Cassette

Construct Containing Three T7 RNA Promoters

The various steps used in this example are depicted in Figure 45. The polylinker from plasmid LIT 28 (New England Biolabs, Inc.) was transferred into an M13 vector by digestion of the plasmid with Bgl II and Hind III and then ligating it with mp18 (New England Biolabs, Inc.) previously digested with Bam H1 and Hind III to create the phage vector LIT Ø1. The plasmid pTS-B (described in Example 19) containing a T7 promoter, the "B" Anti-Sense sequence and the T7 terminator, was digested with EcoRV and Hind III and then ligated to LIT Ø1 previously digested with EcoRV and Hind III to create TOP 302, a phage vector with the "B" Anti-sense T7 operon.

The polylinker from plasmid LIT 38 (New England Biolabs, Inc.) was transferred into an M13 vector by digestion of the plasmid with Spe I and Sph I and then ligating it with mp18 previously digested with Xba I and Sph I to create the phage vector LIT Ø2. The plasmid pTS-A (Example 19) containing a T7 promoter, the "A" anti-sense sequence and the T7 terminator, was digested with EcoRV and Pst I and then ligated to LIT Ø2 previously digested with EcoRV and Pst I to create TOP 414, a phage vector with the A Anti-sense T7 operon. The T7 operons in TOP 302 and TOP 414 were joined together by digestion of TOP 302 with Mlu I and Bsi W1 and ligating it to TOP 414 previously digested with Mlu I and Bsr G1 to form TOP

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501, a phage vector which has both the "A" Anti-Sense T7 operon and the "B" Anti-Sense T7 operon.

The plasmid pTS-C (described in Example 19) containing a T7 promoter, the "C" anti-sense sequence and the T7 terminator, was digested with Sph I and Hind III. TOP 501 was then digested with SphI and Hind III and ligated to pTS-C2 to create TRI 101 which has the "A" Anti-Sense T7 operon, the "B" Anti-Sense T7 operon and the "C" Anti-Sense T7 operon in a single construct. The ordering of the three operons in the TRI 101 construct is given in Figure 46. Co-transfection of this construct with a vector that expresses T7 RNA polymerase (The Intron containing T7 RNA Polymerase described in Example 19 could be used for this purpose) allows the *in vivo* production of all three Anti-Sense transcripts.

Example 29 Construction of an Antisense Expressing Multi-Cassette

Construct Containing Three T7 RNA Promoters and an Intron-
Containing T7 RNA Polymerase Gene.

Although the preceding example utilises the common method of expressing T7 directed transcripts by means of cotransfection with a construct with the RNA polymerase and a second construct with a T7 promoter, an application of the current invention describes a method of carrying both entities (polymerase and promoter) on the same construct. The present example is an illustration of a single construct that contains the T7 RNA polymerase as

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well as multiple operons of T7 driven Anti-Sense transcripts. The various steps used in this example are depicted in Figure 47. The plasmid pTS-C (described above) was digested with EcoRV and Pst I and ligated into the M13 vector LIT Ø2 (described above) which had previously been digested with EcoRV and Pst I, to create the TOP 601 which is a phage vector with the "C" Anti-Sense T7 operon. As described earlier, the construct pINT-3 contains the T7 RNA Polymerase with an SV40 intron inserted within the coding region; in eucaryotic cells there is expression by an RSV promoter followed by excision of the intron by means of the normal splicing machinery of the cell. To insert the T7 Anti-sense operons, it was digested with Spe I and Not I. The T7 Anti-sense operons were inserted as a triple insert by the simultaneous ligation of the Spe/Not pINT-3 DNA with TOP 601 previously digested with Pst I and Nhe I, TOP 302 previously digested with Mlu I and Nsi I and TOP 414 previously ligated with Bpu 120 1 and Mlu I. The resultant clone as well as a diagram of the positions of the different Anti-sense operons is shown in Figure 47.

Example 30 Testing the anti HIV U1 constructs in cells:

Inhibition of virus growth:

a) Creation of stable transformed cell lines:

U937 cells (Laurence, et al., 1991, J. Virol. 65: 214-219) were transformed with the various U1 constructs described above using

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Lipofectin (BRL Inc.) and following the manufacturer's suggested protocol. After transformation, the cultured cells were divided into 2 portions. One portion was used to obtain individual clones while the other portion was used to obtain a population of pooled clones. To obtain the individual clones, aliquots of 1×10^4 cells were seeded into separate chambers in 96 well tissue culture plates and stable transformants were selected by growth in DMEM (Gibco and BRL) medium supplemented with 10% fetal bovine serum (heat inactivated) (Gibco and BRL) in the presence of 600 microgram/ml G418 (Gibco and BRL). The G418-containing medium was replaced every 3 to 4 days, and after 3 weeks of incubation, drug resistant cells were removed from individual wells by aspiration and expanded by growth in culture dishes. To obtain the population of pooled clones, 1×10^6 cells were seeded into T-25 flasks (Corning) and grown in the presence of G418.

b) Characterization of cell lines

RNA was isolated from either resistant clones or resistant pooled clones using hot phenol extraction (Soeiro and Darnell, 1969, J. Mol Biol 44: 551-562). This RNA was used in a dot blot analysis using the protocol accompanying the Genius System (Boehringer Mannheim). The probe used in this analysis was a riboprobe made from a clone of the three inserts (A, B, and C) in pBlueScript (Stratagene) cloned into

the XmaI and BamH1 site. This clone produced insert RNA of the sense orientation. The results of this analysis showed that all cell populations that had been transformed by the U1 clone and that had demonstrated resistance to G418 that were tested expressed the antisense insert RNA. Comparable dot blot analyses were performed using RNA from the parental line U937 as well as yeast RNA (Boehringer Mannheim.) These dots showed no evidence of the antisense insert RNA. The antisense RNA synthesized in vitro using the clones pBlueScript 12, pBlueScript 34, pBlueScript 56 and pBlueScript 78, described above, showed positive hybridization using the sense probe described in this paragraph. From this we conclude that those transformed cell populations that were tested were indeed expressing antisense RNA from the HIV virus sequence.

c) HIV challenge experiment number 1:

0.5×10^6 cells of the pooled clones transformed by the triple U1 construct were incubated with HIV virus at a multiplicity of 0.15 pfu of the virus per cell in the presence of $2\mu\text{g/ml}$ of polybrene for 2 hours at 37°C using the procedure of Laurence et al. (1991 J. Virol. 65: 214-219). The cells were then washed, resuspended in 1 ml of culture medium (RPMI 1640 + 10% fetal bovine serum, Flow Labs) and plated in duplicate (0.5 ml per well.) One-half of the culture medium was removed and replaced with fresh medium every 3-4 days. 6 days post infection, samples of these cells were

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tested for the extent of infection by HIV virus using a p24 ELISA antigen capture following the protocol of the manufacturer (DuPont). The control cultures for this experiment were cells transformed by clones not containing antisense sequences to HIV (see above). The results of this experiment are shown in Table 1.

TABLE 1

Sample	[HIV-1], pg/ml		% Inhibition of HIV p24	
	Expt A	Expt B	Expt A	Expt B
2.2.78 pool control	959 \pm 49		--	
1.9.16 pool	780	error	18.7	--
2.10.16 pool	514	554	46.4	42.2

Both of the pooled clone samples showed inhibition of production of p24 when compared to the control clones. In the instance of the pooled clone 2.10.16, the degree of inhibition when compared to the control was close to 50%. This pooled clone population of cells was examined further as described below.

At 18 days after infection, the p24 concentration in the growth medium was determined as described above. The results of this determination are reported in Table 2.

TABLE 2

Sample	[HIV-1], pg/ml	% Inhibition of HIV p24
U937 control	200	--
2.2.78 pool control	220 ± 2	0
2.10.16 pool	12 ± 0.4	94.5

This table shows that there is approximately 95% inhibition of p24 antigen production in the pooled clone population of cells when compared with either the control pooled clone population or the parent cell line.

On day 24 after viral inoculation, when the cells were assayed by trypan blue dye exclusion the control pooled clone population were 17% viable, and contained numerous syncytia (multinucleated giant cells characteristic of HIV infection). The pooled clone population labeled as 2.10.16 were 40-60% viable and had no visible syncytia.

After day 24, the cells of the control pooled clone culture and the pooled clone culture were subjected to ficol gradient separation (Pharmacia). This procedure separates the live cells from the dead cells every 3-4 days as a routine maintenance procedure. At 35 days, there were no cells left in the control pooled clone population of cells, while the pooled clone population had viable cells. When these viable cells from the pooled clone population were then assayed for the presence of the p24 antigen, it was found that the culture line named 2.10.16 showed no evidence of the presence of p24 antigen in the culture medium above the background (0.032 +/- 0.08 OD compared with 0.039 OD). In this experiment, the HIV infected cells had a measured amount of p24 antigen that was greater than 2 OD. Thus by this time in the selection protocol, the degree of inhibition of the virus was greater than 99%.

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d) HIV challenge experiment number 2:

In this experiment, the pooled clone population identified as 2.10.16 (from day 31 of the first challenge) as well as the control pooled clone population and the parent cell line U937 were infected again with the BAL strain of HIV at a multiplicity of 0.10 pfu per cell as described above. After infection, the cells were maintained as described above. At day 9 and day 12 after infection the p24 antigen was determined as described above. The results of this determination are reported in Table 3.

TABLE 3

Cell Type	HIV-1 [p24], pg/ml	
	day 9	day 12
U937	3	5.1 ± 0.4
2.10.16.R1	<1	14.3 ± 1.3

This table shows that at day 12 there is approximately 66% inhibition of p24 antigen production in the pooled clone population of cells when compared with the parent cell line.

When these cells were maintained with separation of the live from the dead cells using the ficol gradient every 3-4 days as described above it

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was found at day 21 that there was no evidence of p24 antigen in the 2.10.16 cell lines when compared with the parental cell line infected with HIV virus. (Here the comparison is of OD units of the 2.10.16 pooled clone population of 0.009 the same number as the control parental line without infection with >2 OD units.)

e) Further characterization of the 2.10.16 cell line after three cycles of challenge with HIV virus:

In this experiment, the pooled clone population identified as 2.10.16R1 (from day 21 of the second challenge experiment) and the parent cell line U937 were infected again with the BAL strain of HIV as described above. After infection, the cells were maintained as described above. On days 14, 27, and 42 after infection, the p24 antigen was determined as described above for the pooled clone population (now called 2.10.16R2) as well as for the parental cell line U937. The results of this determination are reported in Table 4.

TABLE 4		HIV-1 p24							
Sample	Day 9		Day 14		Day 27		Day 43		
	OD	pg/ml	OD	pg/ml	OD	pg/ml	OD	pg/ml	
U937	0.527	122	0.165	25	dead				
2.10.16R2	0.12	0	0.009	0	0.030	0	0.026	0	

TABLE 4 (continued)

buffer	0.013			
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This table shows that by day 27 the parental cells have disappeared from the culture medium. This is consistent with the conclusion that the virus infection has led to the destruction of the cells. In the pooled clone cell population 2.10.16R2, the amount of p24 antigen detected in these supernatants is below the sensitivity of the assay procedure. Thus on the third challenge of the original pooled clone cell population there is no evidence of virus growth.

The parental cell line U937 is known to contain the surface antigen CD4+. This parent strain and the strain 2.10.16R2, pooled strain after 3 cycles of selection, were assayed in a flow cytometer for the presence of the CD4+ antigen by measuring the binding of mouse CD4+ antibody (Becton Dickenson) with fluorescinated goat anti mouse (Tago). As can be seen in Figure 48, CD4+ antigen is present on the surface of the parental strain and the 2.10.16R2 -HIV resistant cell strain. This is evidence that the cells have not been selected to be resistant to infection by HIV virus through the loss of the adsorption protein, specifically the CD4+ antigen.

While the evidence of virus growth based on the production of the gag antigen, p24, demonstrates that the pooled strain of cells containing the genetic antisense does not permit the growth of virus, further evidence that the virus is not present in this cell population was obtained using the DNA PCR assay for the identification of the coding region of the gag gene (the region coding for the p24 antigen) using the standard Cetus primers which detect virtually all HIV-1,-2 isolates (Applied BioSystems). As can be seen from the Figure 49 representing UV illumination of the EtBr stained DNA, the + control (using DNA provided in the kit) gave a band of the expected size (lane 1), while several dilution of the amplification products of 2.10.16R2 DNA did not show such a band.

These data demonstrate that cell lines can be developed using antisense constructs that maintain their CD4 + phenotype. These cell lines do not support the growth of the HIV virus as measured both by the production of the p24 antigen and measured with the quick DNA PCR kit of Cetus. In addition these cell strains have been shown to survive multiple challenge from infectious HIV virus.

Example 31 Testing the anti HIV U1 constructs in cells:

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Inhibition of synthesis of beta-galactosidase activity:

a) Eukaryotic vector carrying target sequence A upstream of the beta-galactosidase gene.

The A segment (from the tar sequence of HIV) of target DNA was isolated as described above. This segment was cloned into the Kpn1 BamH1 site of the eukaryotic vector pSV Lac Z(Invitrogen), that carries Lac Z coding sequences and SV40 enhancer and promoter and poly A signal sequences. The cloning sites is between these sequences. The cloning sequence is diagrammed in the attached figure (figure 50).

b) Expression of beta-galactosidase activity in stably transfected U937 cells:

U937 cells were transformed using the Lipofectin procedure described above. In this experiment positive clones were selected as zeocin resistant. 5 separate transfected cell populations were isolated:

These cells were 1. U937 cells untransfected; 2. U937 cells transformed with the HIV A clone alone; 3. U937 cells transfected with the HIV A clone and then a second time with the U1 antisense A clone (see above for the description of the clone- the second transfection was selected as G418 resistant); 4. U937 cells cotransfected with the HIV A clone and the U1 antisense ABC clone (again see above for a description of the clone); and 5. U937 cells

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These data demonstrate that the production of the enzyme beta-galactosidase that is shown as a blue stain in cell line 2 with the HIV A clone alone or in cell line 5 where both the HIV A clone and the null DNA control is not seen when either the antisense U1 A clone is

cotransfected with the HIV A clone (cell line 3) or the antisense U1 ABC clone is cotransfected with the HIV A clone (cell line 4). Thus the presence of the antisense A sequence in the cell lines with this HIV A clone expressing the enzyme beta-galactosidase blocks the production of this enzyme.

c. Expression of beta-galactosidase activity in extracts:

To measure enzyme activity by soluble assay (Figure 51, upper set of data) extracts were prepared from loge-phase cultures either by sonication or repeated freeze-thawing. The log-phase cells (5×10^6 cells per ml) were washed free of medium with PBS containing 10 mM Mg^{++} and 1 mM Ca^{++} . The washed cells were suspended in 250 mM Tris-Cl, pH 7.5 and freeze-thawed 3 times or alternatively sonicated 5 minutes at maximum output. The crude lysate was centrifuged and enzyme activity was measured in clear supernatants by hydrolysis of the lactose analog ONPG (Sigma). When this substrate is cleaved by the enzyme to make ONP a yellow colored compound produced. Thus the beta-galactosidase activity can be monitored by observing the change in absorbance at 420nm. Extracts prepared from cells that are stably transfected with the HIV A clone produce a yellow color in 30 minutes at $37^{\circ}C$, whereas the extracts prepared from untransfected cells remain colorless even after incubation over night.

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The 5 transfected cell lines were assayed using this soluble assay format and the results are reported in table 5. From this table it can be seen that the U1 anti-A transfected cells do not have measurable amounts of beta-galactosidase activity. (Compare line 3 with lines 2 and 5.) Also it can be seen that the U1 anti-ABC clones do not show measurable amounts of beta-galactosidase activity. (Compare line 4 with lines 2 and 5.) These results confirm the results from the in situ assay of the affect of the U1 anti A and anti ABC clones on the production of beta-galactosidase activity of clones that have the A target cloned into their sequences.

Example 32

Asymptomatic HIV positive patients are given pre-treatment evaluations including medical histories; physical examinations, blood chemistries including CBCs, differential counts, platelet counts; blood chemistries including glucose, calcium, protein, albumin, uric acid, phosphate; Blood Urea Nitrogen and creatinine; Urinalysis; electrocardiogram and chest X-ray; p24 antigen level; CD4 counts; PCR to determine viral load. The p24 antigen, CD4 counts and PCR are done at weekly intervals for 4 weeks prior to removal of cells in order to establish baseline data, and these assays are continued biweekly throughout the period of treatment.

Blood is removed from patients and the peripheral blood mononuclear cells are separated from erythrocytes and neutrophils by Ficoll-Hypaque centrifugation. After washing, the PBMCs are depleted of CD8+ cells by the use of murine anti-human CD8-coated flasks (CELLector™ Flasks, Applied Immune Sciences). Cells which do not adhere to the surface of the flasks are cells assayed for cellular phenotype by flow cytometry and then activated with OKT3 antibody in serum-free medium.

The OKT3-activated cells are resuspended at a concentration of $1-2 \times 10^5$ cells/ml in fresh medium containing 60 units/ml of IL-2. The cells are expanded to about 2×10^6 /ml.

A retrovirus vector containing sequences for the expression of antisense RNA directed at HIV is grown in a packaging cell line. A DNA construct (described in Example F1) is introduced into retrovirus vector LNL6, which contains a neomycin resistance marker. The cells are transduced by resuspension in culture medium to a concentration of approximately 10^5 cells/ml and mixing with culture supernatant from the retrovirus vector infected cells to provide an MOI of approximately 1.0. Five mg/ml protamine sulfate are added and the mixture is incubated at 37°C for 6 hours. The cells are washed three times and placed in G418 containing culture medium.

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This transduction procedure is repeated daily for three consecutive days.

After 7 days in G418 selection medium the G418 is removed and the cells are expanded in the presence of growth factors (as described above). When sufficient cells are produced, they are harvested, washed and resuspended in physiological saline for infusion into the patient. Cellular phenotype is measured by flow cytometry measurements.

This antisense treatment is supplemented by treatment with soluble CD4 protein. Administration commences immediately after the administration of HIV therapy according to the method of (Husson et al., 1992).

This supplemented gene therapy is further supplemented by concurrent administration of AZT.

Many obvious variations might be suggested to those of ordinary skill in the art in light of the above detailed description of the invention. All such variations are fully embraced by the scope and spirit of the present invention as set forth in the claims which now follow.

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WHAT IS CLAIMED IS:

1. A non-naturally occurring construct which when present in a cell produces a product, said construct comprising at least one modified nucleotide, a nucleotide analog or a non-nucleic acid entity, or a combination of the foregoing.
2. The construct of claim 1, wherein said construct or a portion thereof is linear, circular or branched.
3. The construct of claim 1, wherein said construct or a portion thereof is single-stranded, double-stranded, partially double-stranded or triple-stranded.
4. The construct of claim 3, having at least one terminus, said terminus comprising a polynucleotide tail.
5. The construct of claim 4, wherein said polynucleotide tail is hybridized to a complementary polynucleotide sequence.
6. The construct of claim 1, wherein said construct comprises DNA, RNA, a DNA-RNA hybrid, a DNA-RNA chimera, or a combination of the foregoing.

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7. The construct of claim 1, wherein said modified nucleotide has been chemically modified.
8. The construct of claim 6, wherein said chemical modification has been effected to a moiety independently selected from a base, a sugar, and a phosphate, or a combination thereof.
9. The construct of claim 1, wherein at least one of said nucleotide analog or analogs have been modified on the backbone or sidechain or both.
10. The construct of claim 1, wherein said non-nucleic acid entity is attached to a single strand or to both strands of said sequence segment.
11. The construct of claim 1, wherein said non-nucleic acid entity or entities are selected from a natural or synthetic polymer, and a natural or synthetic ligand, or a combination thereof.
12. The construct of claim 11, wherein said natural polymer comprises a modified or unmodified member selected from a polypeptide, a protein, a polysaccharide, a fatty acid, and a fatty acid ester, or a combination of the foregoing.

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13. The construct of claim 11, wherein said synthetic polymer comprises a homopolymer or heteropolymer.

14. The construct of claim 13, wherein said homopolymer or heteropolymer carries a net negative charge or a net positive charge.

15. The construct of claim 1, wherein said construct exhibits a further biological activity imparted by said modified nucleotide, said nucleotide analog, said nucleic acid entity, a ligand, or a combination of the foregoing.

16. The construct of claim 15, wherein said biological activity is selected from nuclease resistance, cell recognition, cell binding, and cellular or nuclear localization, or a combination of the foregoing.

17. The construct of claim 1, further comprising at least one ligand attached covalently or noncovalently to said modified nucleotide or nucleotides, nucleotide analog or analogs, non-nucleic acid entity or entities, or a combination thereof.

18. The construct of claim 17, wherein said ligand or ligands are attached to a single stranded segment, a double stranded segment, a single stranded construct tail, or a sequence complementary to a construct tail, or a combination of the foregoing.

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25. The construct of claim 24, wherein an antibody is bound to said hybridized polynucleotide tail sequences.

26. The construct of claim 25, wherein said antibody comprises a polyclonal or monoclonal antibody.

27. A composition comprising:

(a) a non-natural entity which comprises:

at least one domain to a nucleic acid component; and

at least one domain to a cell of interest; and

(b) said nucleic acid component;

wherein the domain or domains to said nucleic acid component are different from the domain or domains to said cell.

28. The composition of claim 27, wherein said entity comprises a binder.

29. The composition of claim 28, wherein said binder and said domain are the same.

30. The composition of claim 28, wherein said binder and said domain are different.

31. The composition of claim 28, wherein said binder is selected from a polymer, a matrix, a support, or a combination of any of the foregoing.

32. The composition of claim 27, wherein said nucleic acid component is selected from a nucleic acid, a nucleic acid construct, a nucleic acid conjugate, a

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virus, a viral fragment, a viral vector, a viroid, a phage, a plasmid, a plasmid vector, a bacterium and a bacterial fragment, or a combination of the foregoing.

33. The composition of claim 27, wherein said cell is prokaryotic or eukaryotic.

34. The composition of claim 27, wherein said domains are attached covalently or noncovalently, or through a binder, or a combination thereof.

35. The composition of claim 34, wherein said noncovalent binding is selected from ionic interactions and hydrophobic interactions, or a combination thereof.

36. The composition of claim 35, wherein said noncovalent binding comprises a specific complex.

37. The composition of claim 36, wherein said specific complex is mediated by a ligand binding receptor.

38. The composition of claim 37, wherein said ligand binding receptor is selected from a polynucleotide sequence to be recognized by its complementary sequence, an antigen to be recognized by its corresponding monoclonal or polyclonal antibody, an antibody to be recognized by its corresponding antigen, a lectin to be recognized by its corresponding sugar, a hormone to be recognized by its receptor, a receptor to be recognized by its hormone, an inhibitor to be recognized by its enzyme, an enzyme to be recognized by its inhibitor, a cofactor to be recognized by its cofactor enzyme binding site, a cofactor enzyme binding site to be recognized by its cofactor, a binding ligand to be recognized by its substrate, or a combination of the foregoing.

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39. The composition of claim 28, wherein the domain to said nucleic acid component and the domain to said cell of interest are natural, and said binder is attached to said nucleic acid component by means other than a natural binding site.

40. The composition of claim 39, wherein said binder comprises modified fibronectin or modified polylysine, or both.

41. The composition of claim 27, wherein said cell of interest is contained within an organism.

42. The composition of claim 27, further comprising said cell of interest.

43. A method of introducing a nucleic acid component into a cell comprising:

- (a) providing the composition of claim 27; and
- (b) administering said composition.

44. The method of claim 43, wherein administering is carried out *in vivo*.

45. The method of claim 43, wherein administering is carried out *ex vivo*.

46. A kit for introducing a nucleic acid component into a cell of interest, comprising in packaged containers or combination:

- (a) a non-natural entity which comprises at least one domain to said nucleic acid component, and a domain to said cell of interest;
- (b) a nucleic acid component, optionally with

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(c) buffers and instructions.

47. A composition comprising:

an entity which comprises at least one domain to a cell of interest,
wherein said domain or domains are attached to a nucleic acid
component which is in non-double stranded form.

48. The composition of claim 47, wherein said entity comprises a binder.

49. The composition of claim 48, wherein said binder and said domain are the same.

50. The composition of claim 48, wherein said binder and said domain are different.

51. The composition of claim 48, wherein said binder is selected from a polymer, a matrix, a support, or a combination of any of the foregoing.

52. The composition of claim 47, wherein said cell is prokaryotic or eukaryotic.

53. The composition of claim 47, wherein said nucleic acid component is selected from a nucleic acid, a nucleic acid construct, a nucleic acid conjugate, a virus, a viral fragment, a viral vector, a viroid, a phage, a plasmid, a plasmid vector, a bacterium and a bacterial fragment, or a combination of the foregoing.

54. The composition of claim 47, wherein said domain is selected from covalent bonding and noncovalent binding, or a combination thereof.

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55. The composition of claim 54, wherein said noncovalent binding is selected from ionic interactions and hydrophobic interactions, or a combination thereof.

56. The composition of claim 54, wherein said noncovalent binding comprises a specific complex.

57. The composition of claim 56, wherein said specific complex is mediated by a ligand binding receptor.

58. The composition of claim 57, wherein said ligand binding receptor is selected from a polynucleotide sequence to be recognized by its complementary sequence, an antigen to be recognized by its corresponding monoclonal or polyclonal antibody, an antibody to be recognized by its corresponding antigen, a lectin to be recognized by its corresponding sugar, a hormone to be recognized by its receptor, a receptor to be recognized by its hormone, an inhibitor to be recognized by its enzyme, an enzyme to be recognized by its inhibitor, a cofactor to be recognized by its cofactor enzyme binding site, a cofactor enzyme binding site to be recognized by its cofactor, a binding ligand to be recognized by its substrate, or a combination of the foregoing.

59. The composition of claim 47, wherein said cell of interest is contained within an organism.

60. The composition of claim 47, further comprising said cell of interest.

61. A method of introducing a nucleic acid component into a cell comprising:

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- (a) providing the composition of claim 47; and
- (b) administering said composition.

62. The method of claim 61, wherein administering is carried out *in vivo*.

63. The method of claim 61, wherein administering is carried out *ex vivo*.

64. A kit for introducing a nucleic acid component into a cell of interest, comprising in packaged containers or combinations:

- (a) an entity which comprises a domain to said cell of interest, wherein said domain is attached to a nucleic acid component which is in non-double stranded form, optionally with
- (b) buffers and instructions.

65. A composition comprising:

an entity which comprises a domain to a nucleic acid component, wherein said domain is attached to a cell of interest.

66. The composition of claim 65, wherein said entity comprises a binder.

67. The composition of claim 66, wherein said binder and said domain are the same.

68. The composition of claim 66, wherein said binder and said domain are different.

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69. The composition of claim 66, wherein said binder is selected from a polymer, a matrix, a support, or a combination of any of the foregoing.
70. The composition of claim 66, wherein said nucleic acid component is selected from a nucleic acid, a nucleic acid construct, a nucleic acid conjugate, a virus, a viral fragment, a viral vector, a viroid, a phage, a plasmid, a plasmid vector, a bacterium and a bacterial fragment, or a combination of the foregoing.
71. The composition of claim 65, wherein said cell is eukaryotic or prokaryotic.
72. The composition of claim 65, wherein said domain is selected from covalent bonding and noncovalent binding, or a combination thereof.
73. The composition of claim 72, wherein said noncovalent binding is selected from ionic interactions and hydrophobic interactions, or a combination thereof.
74. The composition of claim 72, wherein said noncovalent binding comprises a specific complex.
75. The composition of claim 74, wherein said specific complex is mediated by a ligand binding receptor.
76. The composition of claim 75, wherein said ligand binding receptor is selected from a polynucleotide sequence to be recognized by its complementary sequence, an antigen to be recognized by its corresponding monoclonal or polyclonal antibody, an antibody to be recognized by its corresponding antigen, a lectin to be recognized by its corresponding sugar, a hormone to be recognized

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by its receptor, a receptor to be recognized by its hormone, an inhibitor to be recognized by its enzyme, an enzyme to be recognized by its inhibitor, a cofactor to be recognized by its cofactor enzyme binding site, a cofactor enzyme binding site to be recognized by its cofactor, a binding ligand to be recognized by its substrate, or a combination of the foregoing.

77. The composition of claim 65, further comprising said cell of interest.

78. The composition of claim 65, wherein said cell of interest is contained within an organism.

79. A method of introducing a nucleic acid component into a cell comprising:

- (a) providing the composition of claim 65; and
- (b) administering said composition.

80. The method of claim 79, wherein administering is carried out *in vivo*.

81. The method of claim 79, wherein administering is carried out *ex vivo*.

82. A kit for introducing a nucleic acid component into a cell of interest, comprising in packaged containers or combination:

- (a) an entity which comprises a domain to said nucleic acid component, wherein said domain is attached to said cell of interest, optionally with
- (b) buffers and instructions.

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83. A multimeric complex composition comprising more than one monomeric unit attached.

- (a) to each other through polymeric interactions, or
- (b) to a binding matrix through polymeric interactions, or
- (c) both (a) and (b)

84. The composition of claim 83, wherein the polymer or oligomer of said monomeric unit is linear or branched.

85. The composition of claim 83, wherein the polymer or oligomer of said monomeric unit comprises of homopolymer or heteropolymer.

86. The composition of claim 83, wherein said monomeric unit comprises an analyte-specific moiety.

87. The composition of claim 86, wherein said analyte-specific moiety is capable of recognizing a component in a biological system.

88. The composition of claim 87, wherein said biological system is selected from a virus, a phage, a bacterium, a cell or cellular material, a tissue, an organ and an organism, or a combination thereof.

89. The composition of claim 83, wherein said monomeric unit is selected from a naturally occurring compound, a modified natural compound, a

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95. The composition of claim 83, wherein the polymer or oligomer of said binding matrix is linear or branched.

96. The composition of claim 83, wherein the polymer or oligomer of said binding matrix comprises a homopolymer or heteropolymer.

97. The composition of claim 83, wherein said binding matrix is selected from a naturally occurring compound, a modified natural compound, a synthetic compound and a recombinantly produced compound, or a combination thereof.

98. The composition of claim 83, wherein said binding matrix comprises a member selected from a polypeptide, a polynucleotide and a polysaccharide, or a combination thereof.

99. The composition of claim 83, wherein said polymeric interactions are selected from ionic interactions, hydrogen bonding, dipole-dipole interactions, or a combination of the foregoing.

100. The composition of claim 99, wherein said ionic interactions comprise polycationic interactions or polycationic interactions.

101. The composition of claim 83, further comprising an entity attached to said binding matrix.

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102. The composition of claim 101, wherein said entity comprises a ligand or a compound which increases binding of the binding matrix.

103. The composition of claim 83, in homogeneous form.

104. The composition of claim 83, in heterogeneous form.

105. A process for delivering a cell effector to a cell, comprising:
providing the multimeric complex composition of claim 83 wherein
said monomeric unit comprises said cell effector; and administering
said composition.

106. The process of claim 105, wherein said composition is delivered
in vivo.

107. The process of claim 105, wherein said composition is delivered
ex vivo.

108. The process of claim 105, wherein said cell is contained in an
organism.

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109. A process for delivering a gene or fragment thereof to a cell,
comprising:

providing the multimeric complex composition of claim 83, wherein
said monomeric unit comprises said gene or gene fragment; and
administering said composition.

110. The process of claim 109, wherein said composition is delivered
in vivo.

111. The process of claim 109, wherein said composition is delivered
ex vivo.

112. The process of claim 109, wherein said cell is contained in an
organism.

113. A multimeric composition comprising more than one component
attached to a charged polymer, wherein said charged polymer is selected
from a polycationic polymer, a polyionic polymer, a polynucleotide, a
modified polynucleotide and a polynucleotide analog, or a combination of the
foregoing.

114. The multimeric composition of claim 113, wherein said component
comprises a protein.

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115. The multimeric composition of claim 114, wherein said protein is selected from an antibody and an F(ab')₂ fragment, or both.

116. The multimeric composition of claim 115, wherein said antibody comprises a polyclonal or monoclonal antibody.

117. The multimeric composition of claim 115, wherein said antibody is further complex with a target comprising an enzyme.

118. A nucleic acid construct which when introduced into a cell codes for and expresses a non-native polymerase, said polymerase being capable of producing more than one copy of a nucleic acid sequence from said construct.

119. The construct of claim 118, further comprising a recognition site for said non-native polymerase.

120. The construct of claim 119, wherein said recognition site is complementary to a primer for said non-native polymerase.

121. The construct of claim 120, wherein said primer comprises transfer RNA (tRNA).

122. The construct of claim 118, wherein said non-native polymerase comprises a member selected from DNA polymerase, RNA polymerase and reverse transcriptase, or a combination thereof.

123. The construct of claim 122, wherein said RNA polymerase comprises a bacteriophage RNA polymerase.

124. The construct of claim 123, wherein said bacteriophage RNA polymerase is selected from T3, T7 and SP6, or a combination thereof.

125. The construct of claim 122, further comprising a promoter for said RNA polymerase.

126. The construct of claim 118, wherein said nucleic acid produced from said construct is selected from DNA, RNA, a DNA-RNA hybrid and a DNA-RNA chimera, or a combination of the foregoing.

127. The construct of claim 126, wherein said DNA or RNA comprises sense or antisense, or both.

128. A nucleic acid construct which when introduced into a cell produces a nucleic acid product comprising a non-native processing element, which

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when in a compatible cell, said processing element is substantially removed during processing.

129. The construct of claim 128, wherein said processing element comprises an RNA processing element.

130. The construct of claim 129, wherein said RNA processing element is selected from an intron, a polyadenylation signal and a capping element, or a combination of the foregoing.

131. The construct of claim 128, wherein said nucleic acid product is single stranded.

132. The construct of claim 128, wherein said nucleic acid product is selected from antisense RNA, antisense DNA, sense RNA, sense DNA, a ribozyme and a protein binding nucleic acid sequence, or a combination of the foregoing.

133. The composition of claim 132, wherein said protein binding nucleic acid sequence comprises a decoy that binds a protein required for viral assembly or viral replication.

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134. A process for selectively expressing a nucleic acid product in a cell, which product requires processing for functioning, said process comprising:

- (i) providing a nucleic acid construct which when introduced into a cell produces a nucleic acid product comprising a non-native processing element, which when in a compatible cell, said processing element is substantially removed during processing;
- and (ii) introducing said construct into said cell.

135. The process of claim 134, wherein said processing element comprises an RNA processing element selected from an intron, a polyadenylation signal and a capping element, or a combination of the foregoing.

136. The process of claim 134, wherein said nucleic acid product is selected from antisense RNA, antisense DNA, sense RNA, sense DNA, a ribozyme and a protein binding nucleic acid sequence, or a combination of the foregoing.

137. The process of claim 134, wherein said construct is introduced *ex vivo* into said cell.

138. The process of claim 137, wherein said construct is introduced *in vivo* into said cell.

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139. The process of claim 134, wherein said construct is introduced into a biological system containing said cell.

140. The process of claim 139, wherein said biological system is selected from an organism, an organ, a tissue and a culture, or a combination of the foregoing.

141. A composition comprising a primary nucleic acid component which upon introduction into a cell produces a secondary nucleic acid component which is capable of producing a nucleic acid product, or a tertiary nucleic acid component, or both, wherein said primary nucleic acid component is not obtained with said secondary or tertiary component or said nucleic acid product.

142. The composition of claim 141, wherein said cell is eukaryotic or prokaryotic.

143. The composition of claim 141, wherein said primary nucleic acid component is selected from a nucleic acid, a nucleic acid construct, a nucleic acid conjugate, a virus, a viral fragment, a viral vector, a viroid, a phage, a phage, a plasmid, a plasmid vector, a bacterium and a bacterial fragment, or a combination of the foregoing.

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144. The composition of claim 141, wherein said primary nucleic acid component is single-stranded, double-stranded or partially double-stranded.

145. The composition of claim 141, wherein said primary nucleic acid component is selected from DNA, RNA and nucleic acid analogs, or a combination thereof.

146. The composition of claim 145, wherein said DNA, RNA or both are modified.

147. The composition of claim 141, wherein said secondary nucleic acid component or said tertiary nucleic acid component is selected from DNA, RNA, a DNA-RNA hybrid and a DNA-RNA chimera, or a combination of the foregoing.

148. The composition of claim 141, further comprising a signal processing sequence.

149. The composition of claim 148, wherein said signal processing sequence is selected from a promoter, an initiator, a terminator, an intron and a cellular localization element, or a combination of the foregoing.

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150. The composition of claim 148, wherein said signal processing sequence is contained in an element selected from said primary nucleic acid component, said secondary nucleic acid component, said nucleic acid product and said tertiary nucleic acid component, or a combination of the foregoing.

151. The composition of claim 141, wherein said nucleic acid product is single-stranded.

152. The composition of claim 141, wherein said nucleic acid product is selected from antisense RNA, antisense DNA, a ribozyme and a protein binding nucleic acid sequence, or a combination of the foregoing.

153. The composition of claim 152, wherein said protein binding nucleic acid sequence comprises a decoy that binds a protein required for viral assembly or viral replication.

154. The composition of claim 141, wherein said component or nucleic acid production is mediated by a vector.

155. The composition of claim 154, wherein said vector is selected from a viral vector, a phage vector and a plasmid vector, or a combination thereof.

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156. A cell containing the composition of claim 141.

157. The cell of claim 156, wherein said cell is a eukaryotic or prokaryotic.

158. The cell of claim 156, wherein said composition has been introduced *ex vivo* into said cell.

159. The cell of claim 156, wherein said composition has been introduced *in vivo* into said cell.

160. A secondary or tertiary nucleic acid component or nucleic acid product produced from the composition of claim 1.

161. A composition of matter comprising a nucleic acid component which when present in a cell produces a non-natural nucleic acid product, which product comprises (i) a portion of a localizing localizing entity, and (ii) a nucleic acid sequence of interest.

162. The composition of claim 161, wherein said portion of the localizing entity (i) is sufficient to permit localization of said non-natural nucleic acid product.

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163. The composition of claim 161, wherein said said portion of the localizing entity (i) comprises a cytoplasmic or nuclear localization signalling sequence.

164. The composition of claim 161, wherein said nucleic acid sequence of interest (ii) is selected from DNA, RNA, a DNA-RNA hybrid and a DNA-RNA chimera, or a combination of the foregoing.

165. The composition of claim 164, wherein said RNA comprises a nuclear localized RNA complexed with protein molecules.

166. The composition of claim 165, wherein said nuclear localized RNA comprises a snRNA.

167. The composition of claim 166, wherein said snRNA comprises U1 or U2, or both.

168. The composition of claim 161, wherein said non-natural nucleic acid product is single-stranded.

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	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100
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plasmid, a plasmid vector, a bacterium and a bacterial fragment, or a combination of the foregoing.

175. The composition of claim 174, wherein said nucleic acid is selected from DNA, RNA, a DNA-RNA hybrid and a DNA-RNA chimera, or a combination of the foregoing.

176. The composition of claim 174, wherein said nucleic acid is modified.

177. The composition of claim 161, wherein said cell is eukaryotic or prokaryotic.

178. The composition of claim 161, wherein the production of said nucleic acid product is mediated by a vector.

179. The composition of claim 178, wherein said vector is selected from a viral vector, a phage vector and a plasmid vector, or a combination thereof.

180. A cell containing the composition of claim 161.

181. The cell of claim 180, wherein said cell is eukaryotic or prokaryotic.

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182. The cell of claim 180, wherein said composition has been introduced ex vivo into said cell.

183. The cell of claim 180, wherein said composition has been introduced in vivo into said cell.

184. A biological system containing the cell of claim 180.

185. The biological system of claim 184, wherein said system is selected from an organism, an organ, a tissue and a culture, or a combination thereof.

186. A process for localizing a nucleic acid product in a eukaryotic cell, comprising:

- (a) providing a composition of matter comprising a nucleic acid component which when present in a cell produces a non-natural nucleic acid product, which product comprises:

- (i) a portion of a localizing entity, and

- (ii) a nucleic acid sequence of interest;

and (b) introducing said composition into said cell or into a biological system containing said cell.

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187. The process of claim 186, wherein said portion of the localizing entity (I) is sufficient to permit localization of said nucleic acid product.

188. The process of claim 186, wherein said nucleic acid product comprises antisense RNA or antisense DNA and said portion of a localizing entity (I) comprises a nuclear localization signalling sequence.

189. The process of claim 186, wherein said nucleic acid product comprises sense RNA or sense DNA and said portion of a localizing entity (i) comprises a nuclear localization signalling sequence.

190. The process of claim 186, wherein said nucleic acid product comprises sense RNA or sense DNA and said portion of a localizing entity (I) comprises a nuclear localization signalling sequence.

191. The process of claim 186, wherein said nucleic acid product comprises snRNA.

192. The process of claim 191, wherein said snRNA comprises U1 or U2 or both.

193. The process of claim 186, wherein said composition is introduced

ex vivo into said cell or into a biological system containing said cell.

194. The process of claim 186, wherein said composition is introduced *in vivo* into said cell or into a biological system containing said cell.

195. A nucleic acid component which upon introduction into a cell is capable of producing more than one specific nucleic acid sequence, each such specific sequence so produced being substantially nonhomologous with each other and being either complementary with a specific portion of a single-stranded nucleic acid of interest in a cell or capable of binding to a specific protein of interest in a cell.

196. The nucleic acid component of claim 195, wherein said single-stranded nucleic acids of interest are part of the same polynucleotide sequence or part of different polynucleotide sequences.

197. The nucleic acid component of claim 195, wherein said single-stranded nucleic acids of interest comprise a viral sequence.

198. The nucleic acid component of claim 195, wherein said component is derived or selected from a nucleic acid, a nucleic acid construct, a nucleic acid conjugate, a virus, a viral fragment, a viral vector, a phage, a plasmid, a bacterium and a bacterial fragment, or a combination of any of the foregoing.

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204. The nucleic acid component of claim 203, wherein said complementary specific nucleic acid sequence products are capable of acting as antisense.

205. The nucleic acid component of claim 204, wherein said viral or cellular protein comprises a localizing protein or a decoy protein.

206. The nucleic acid component of claim 205, wherein said localizing protein comprises a nuclear localizing protein or a cytoplasmic localizing protein.

207. The nucleic acid component of claim 205, wherein said decoy protein binds a protein required for viral assembly or viral replication.

208. The nucleic acid component of claim 195, wherein said specific nucleic acid sequence products are selected from antisense RNA, antisense DNA, a ribozyme and a protein binding nucleic acid sequence, or a combination of any of the foregoing.

209. The nucleic acid component of claim 195, further comprising a means for delivering said component to a cell containing the nucleic acid of interest or the specific protein of interest.

210. A process for increasing cellular resistance to a virus of interest, comprising:

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- (a) providing:
 - (i) transformed cells phenotypically resistant to said virus; and
 - (ii) a reagent capable of binding to said virus or to a virus-specific site on said cells;
- and (b) administering said reagent to a biological system containing said cells to increase the resistance of said cells to the virus of interest.

211. The process of claim 210, wherein said biological system is selected from an organism, an organ and a tissue, or a combination thereof.

212. The process of claim 210, wherein said viral resistant cells (i) are eukaryotic or prokaryotic.

213. The process of claim 210, wherein said viral resistant cells (i) comprise a nucleic acid sequence selected from antisense RNA, antisense DNA, sense RNA, sense DNA, a ribozyme and a protein binding nucleic acid sequence, or a combination of the foregoing.

214. The process of claim 210, wherein said virus binding reagent (ii) is selected from an antibody, a virus binding protein, a cell receptor protein and

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an agent capable of stimulating production of a virus binding protein, or a combination of the foregoing.

215. The process of claim 214, wherein said antibody comprises a polyclonal or monoclonal antibody.

216. The process of claim 215, wherein said polyclonal or monoclonal antibody is specific to an epitope of said virus of interest.

217. The process of claim 214, wherein said virus binding protein comprises a CD4 receptor.

218. The process of claim 214, wherein said cell receptor protein comprises a gp24 protein.

219. The process of claim 214, wherein said production stimulating agent is selected from an immunological response enhancing adjuvant and a viral antigen, or a combination of both.

220. The process of claim 210 wherein said reagent (ii) is administered *in vivo* to said cells.

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221. The process of claim 210, wherein said reagent (ii) is administered *ex vivo* to said cells.

222. The process of claim 210, further comprising administering an additional viral resistance enhancing agent (iii).

223. The process of claim 222, wherein said additional viral resistance enhancing agent (iii) is selected from a protease inhibitor, a nucleoside analog, or a combination thereof.

224. The process of claim 222, wherein said additional viral resistance enhancing agent (iii) is administered before administering said binding reagent (ii).

225. The process of claim 222, wherein said additional viral resistance enhancing agent (iii) is administered after administering said binding reagent (ii).

226. The process of claim 222, wherein said additional viral resistance enhancing agent (iii) is administered at about the same time that said binding reagent (ii) is administered.

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227. A biological system with increased viral resistance obtained by the process of claim 210.

228. A biological system with increased viral resistance obtained by the process of claim 222.

229. A nucleic acid construct which when introduced into a cell produces a non-natural product, which product comprises two components:

- (i) a binding component capable of binding to a cellular component;
- and (ii) a localization component capable of dislocating said cellular component when bound to said product.

230. The construct of claim 229, wherein said product comprises a protein or a nucleic acid, or a combination of both.

231. The construct of claim 230, wherein said protein comprises an antibody.

232. The construct of claim 231, wherein said antibody comprises a polyclonal or monoclonal antibody.

233. The construct of claim 232 wherein said polyclonal or monoclonal antibody is directed to a cellular component inside the cell.

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239. The construct of claim 237, wherein said protein is selected from an antibody, a receptor and a nucleic acid binding protein, or a combination of the foregoing.

240. The construct of claim 237, wherein said binding entity is capable of binding metabolites.

241. The construct of claim 229, wherein said localization component is selected from a nuclear localizing entity, a cytoplasmic localizing entity and a cell membrane localizing entity, or a combination thereof.

242. The construct of claim 229, wherein said localization component comprises a member selected from a nucleic acid sequence, a nucleic acid structure and a peptide or oligopeptide, or a combination of the foregoing.

243. The construct of claim 242, wherein said nucleic acid structure comprises a stem and loop structure.

244. A process for dislocating a cellular component in a cell, comprising:

(I) providing:

(a) a nucleic acid construct which when introduced into a cell produces a non-natural product, which product comprises two components:

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- (i) a binding component capable of binding to a cellular component; and
 - (ii) a localization component capable of dislocating said cellular component when bound to said product;
- and (II) introducing said nucleic acid construct into a cell of interest or a biological system containing said cell of interest.

* * * * *

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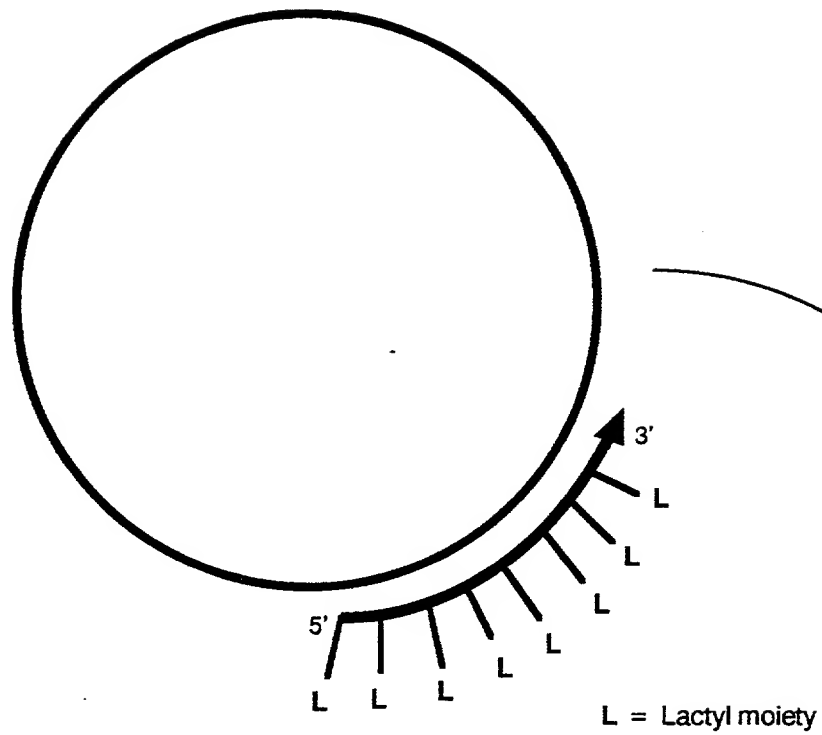
ABSTRACT OF THE DISCLOSURE

**NOVEL PROPERTY EFFECTING AND/OR PROPERTY EXHIBITING
COMPOSITIONS FOR THERAPEUTIC AND DIAGNOSTIC USES**

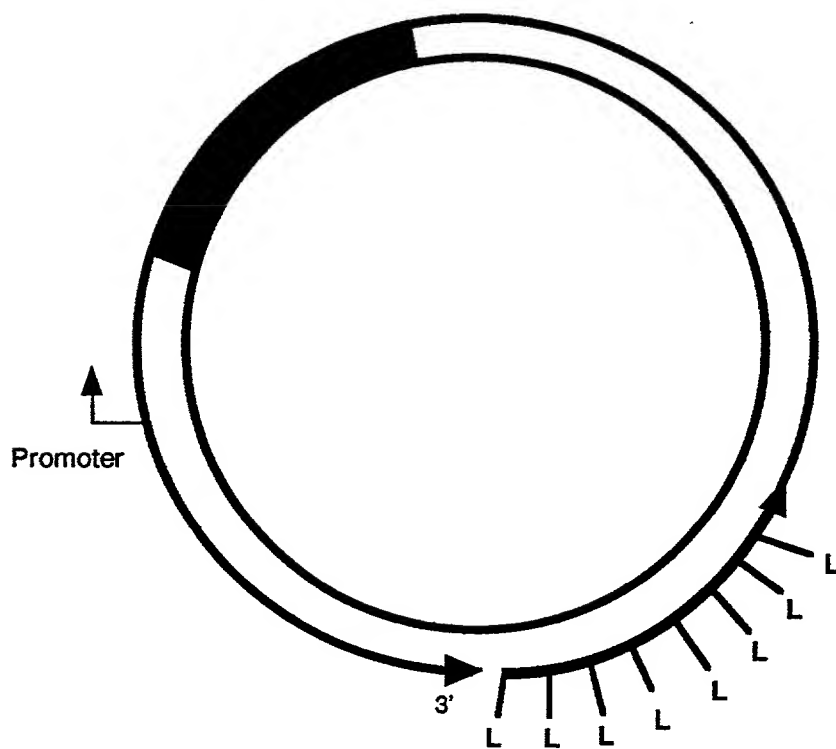
The present invention provides an array of compositions useful for effecting and/or exhibiting changes in biological functioning and processing within cells and in biological systems containing such cells. In effect, these compositions combine chemical modifications and/or ligand additions with biological functions. The chemical modifications and/or ligand additions provide additional characteristics to the compositions without interfering substantially with their biological function. Such additional characteristics include nuclease resistance, targeting specific cells or specific cell receptors localizing to specific sites within cells and augmenting interactions between the compositions and target cells of interest as well as decreasing such interactions when desired. Also provided by the present invention are processes and kits.

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(a)



(b)

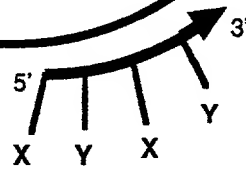
**Figure 1**

Attachment of Ligands Through Primer Region

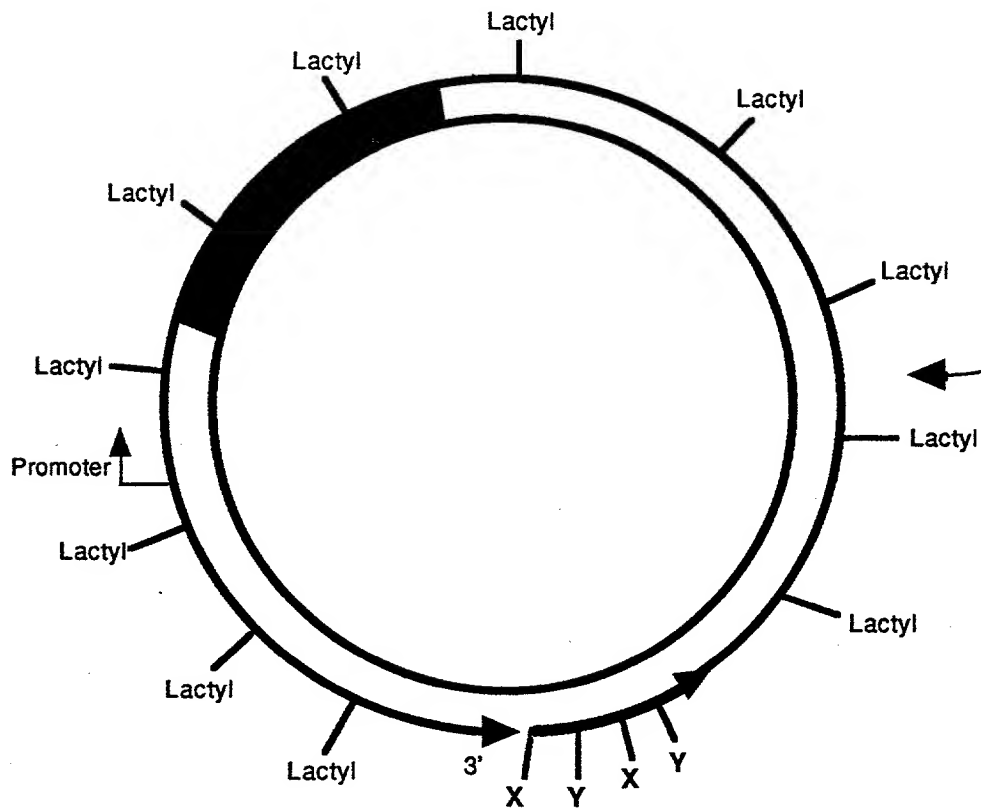
(a)

X = Nuclear Localisation Signal

Y = fusogenic peptide



(b)

**Figure 2**

Attachment of Ligands by Incorporation of
Modified Nucleotide Precursors

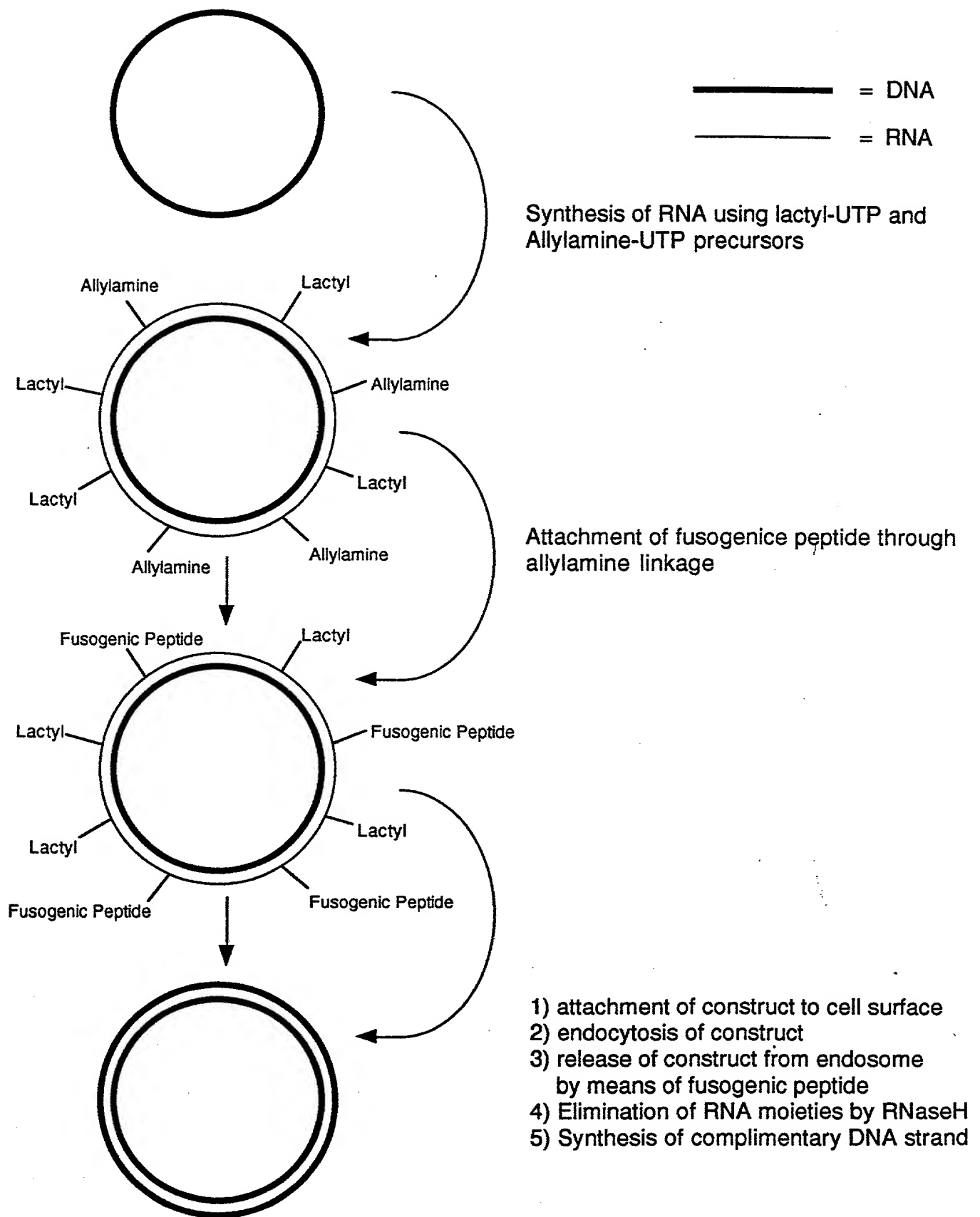


Figure 3
Incorporation of Ligands through Modified Ribonucleotides

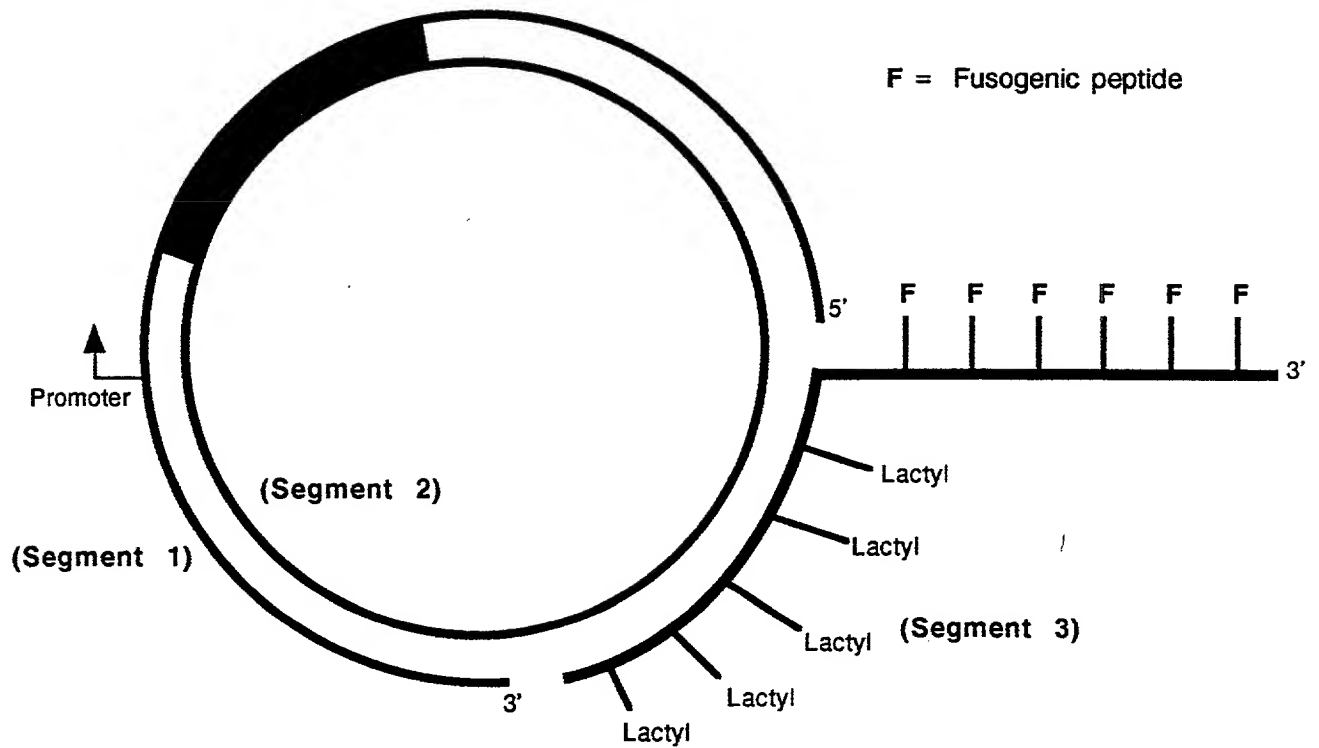


Figure 4

Attachment of Ligands through a 3' tail

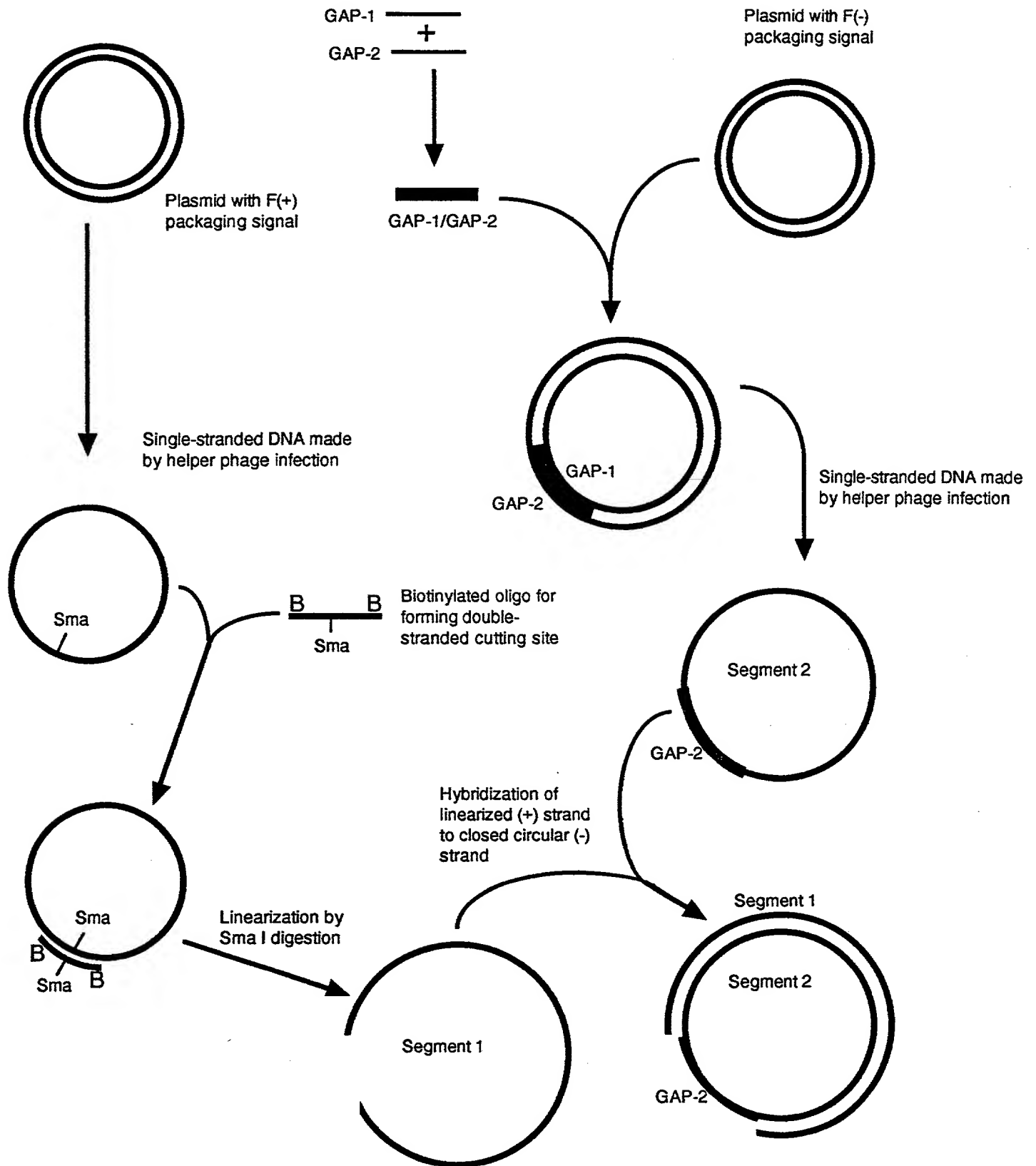
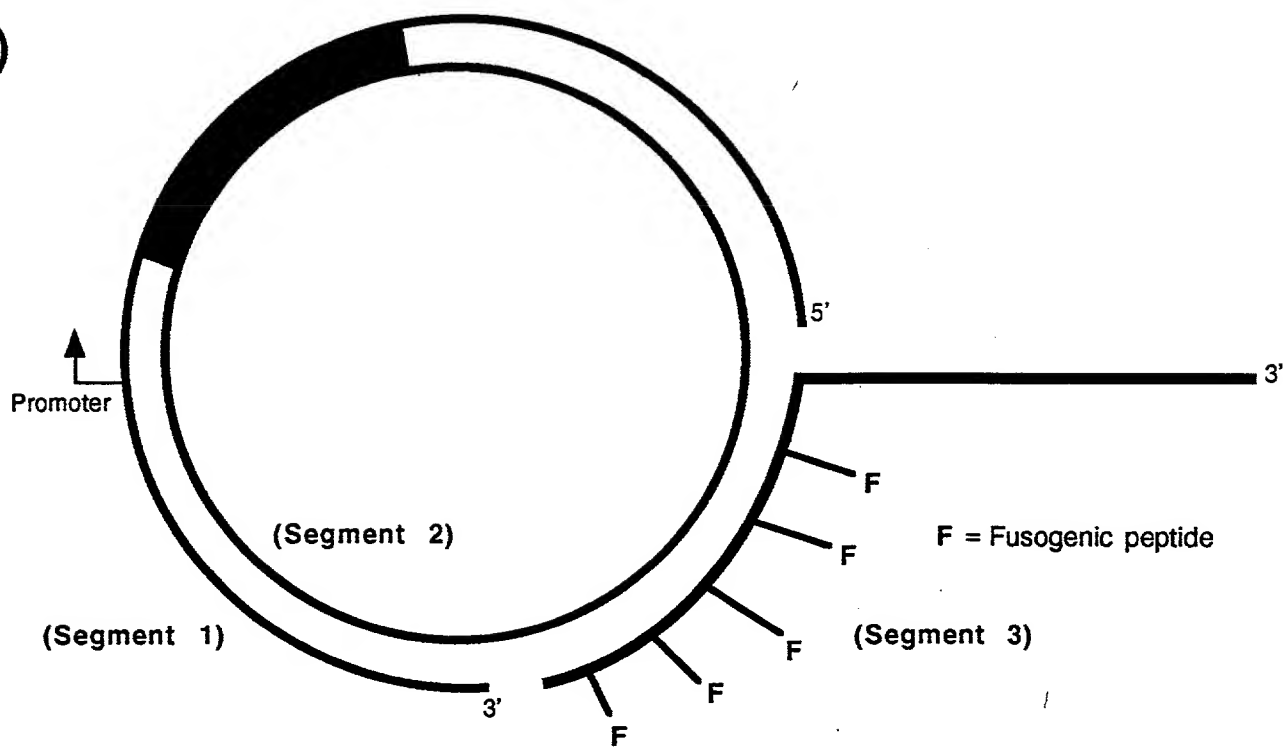
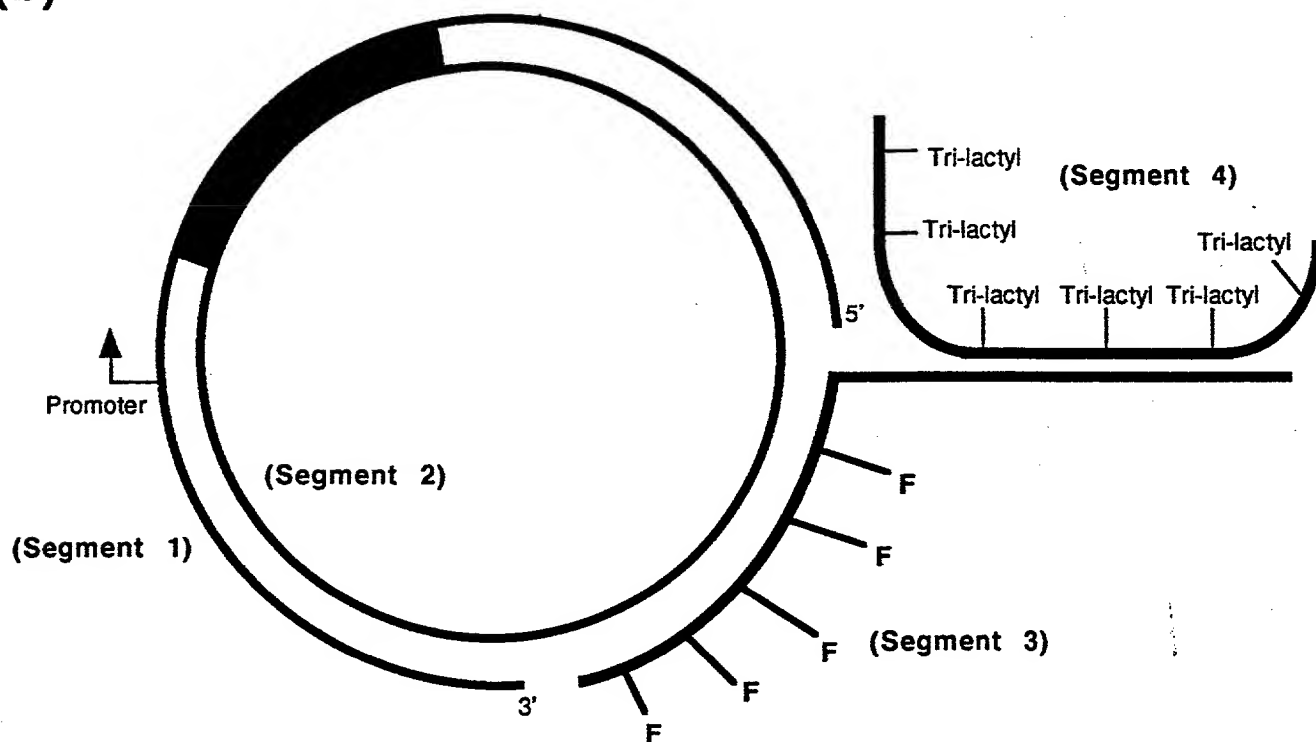


Figure 5
Preparation of Gapped Circle

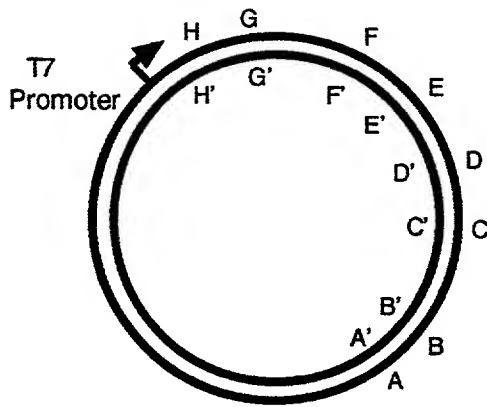
(a)



(b)

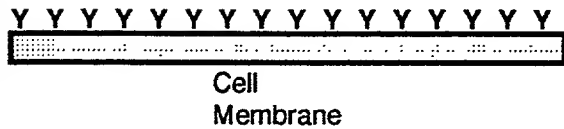
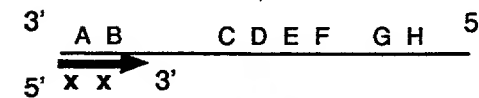
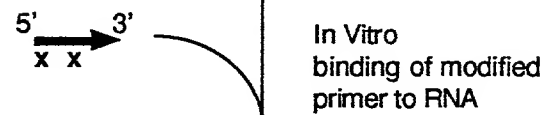
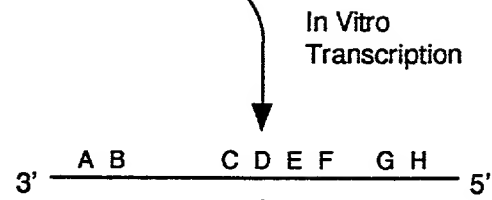
**Figure 6**

Attachment of Ligands through hybridization to a 3' tail

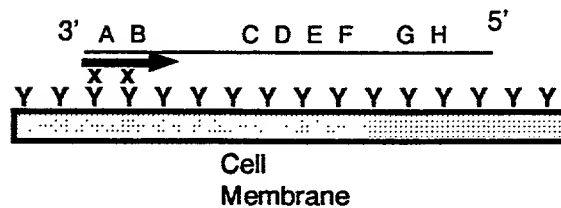


X = Ligands attached to DNA
Y = Ligand Receptors on cell

— = DNA
— = RNA



Binding of nucleic acids to membrane through ligands on Primer



Endocytosis

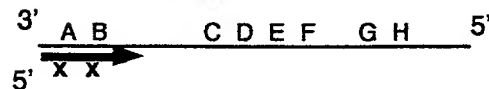
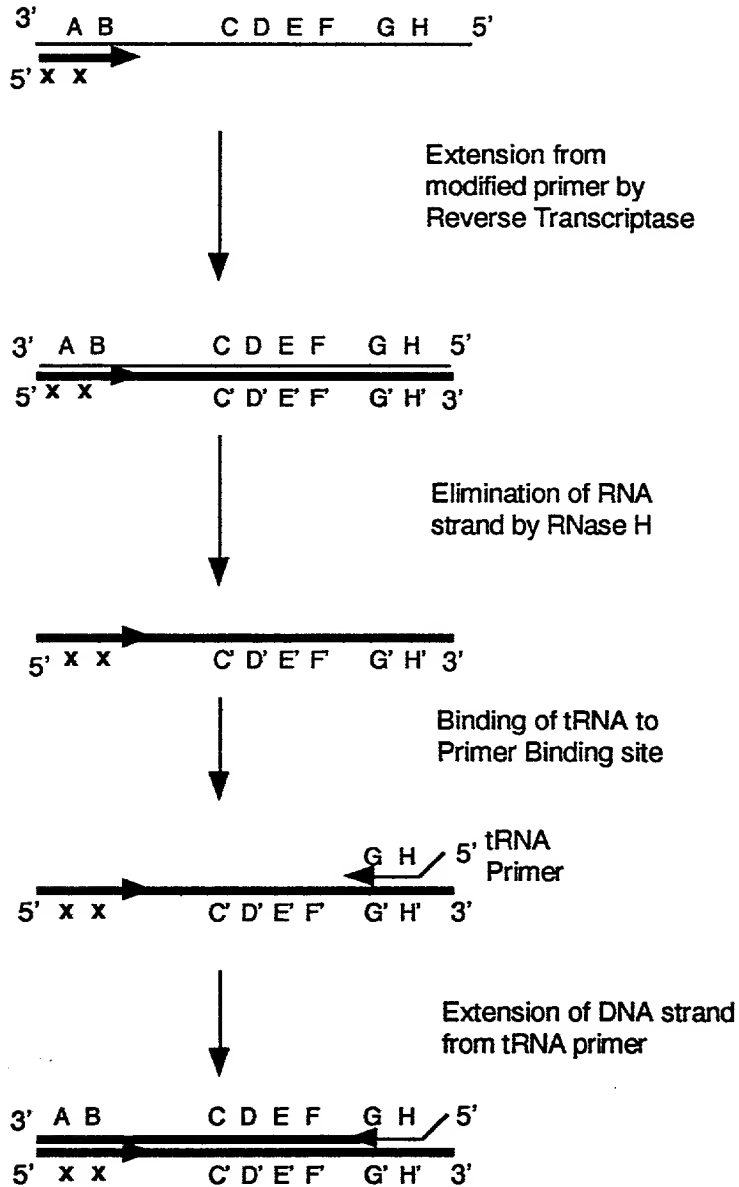


Figure 7

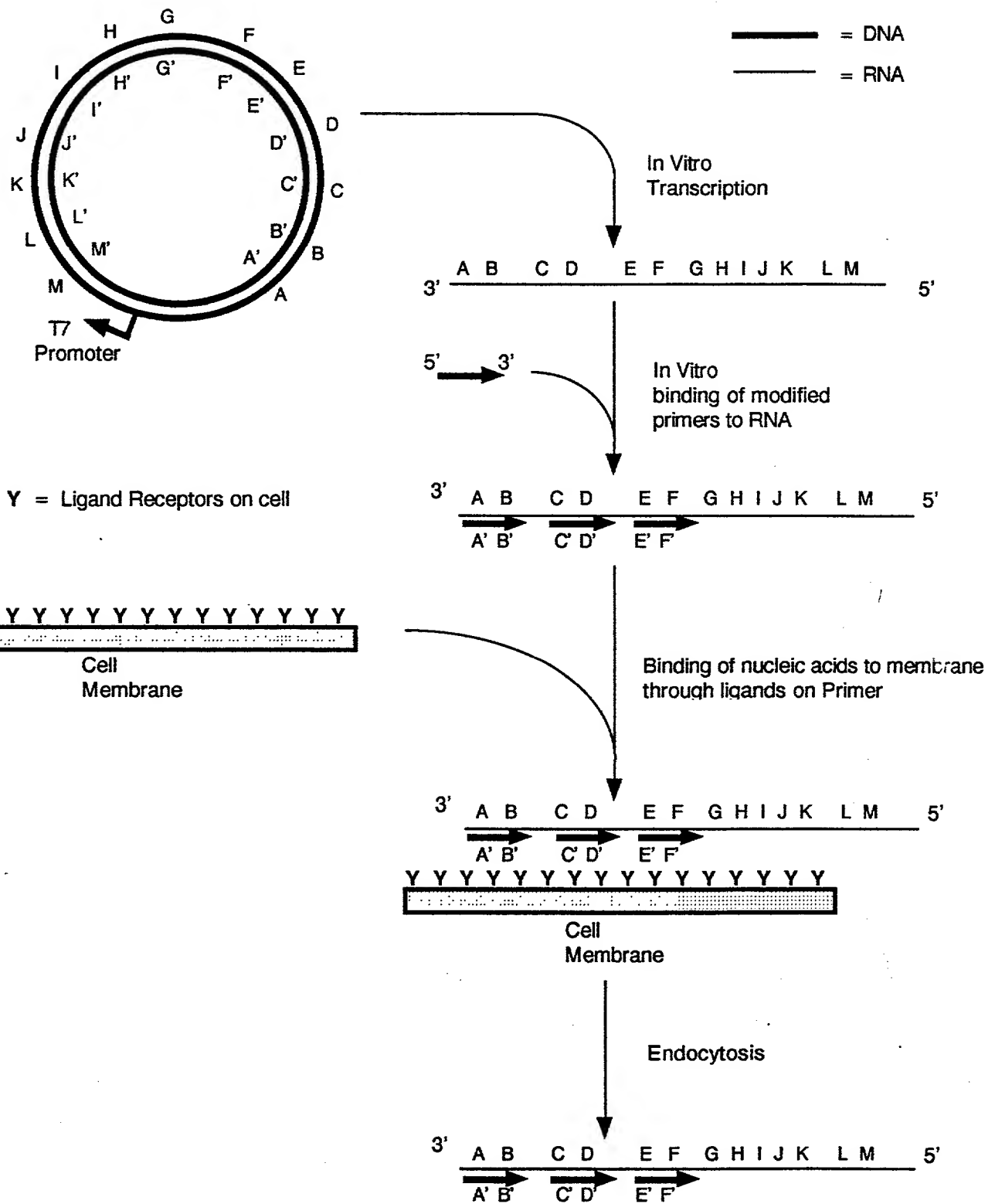
RNA with Ligands on Primer

(Continued in Figure 8)

Continued from Figure 7

**Figure 8**

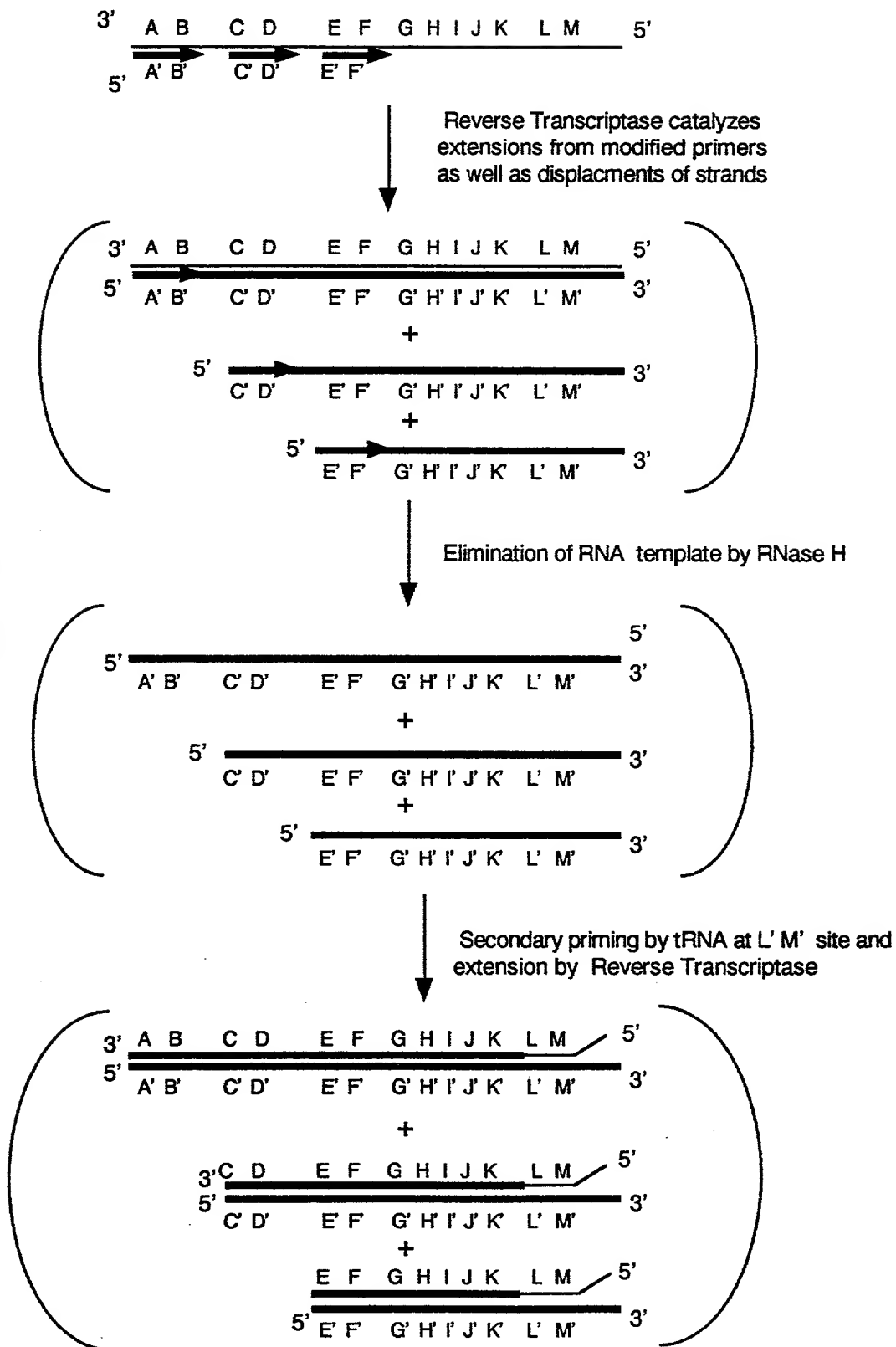
RNA with Ligands on Primer (Continued)



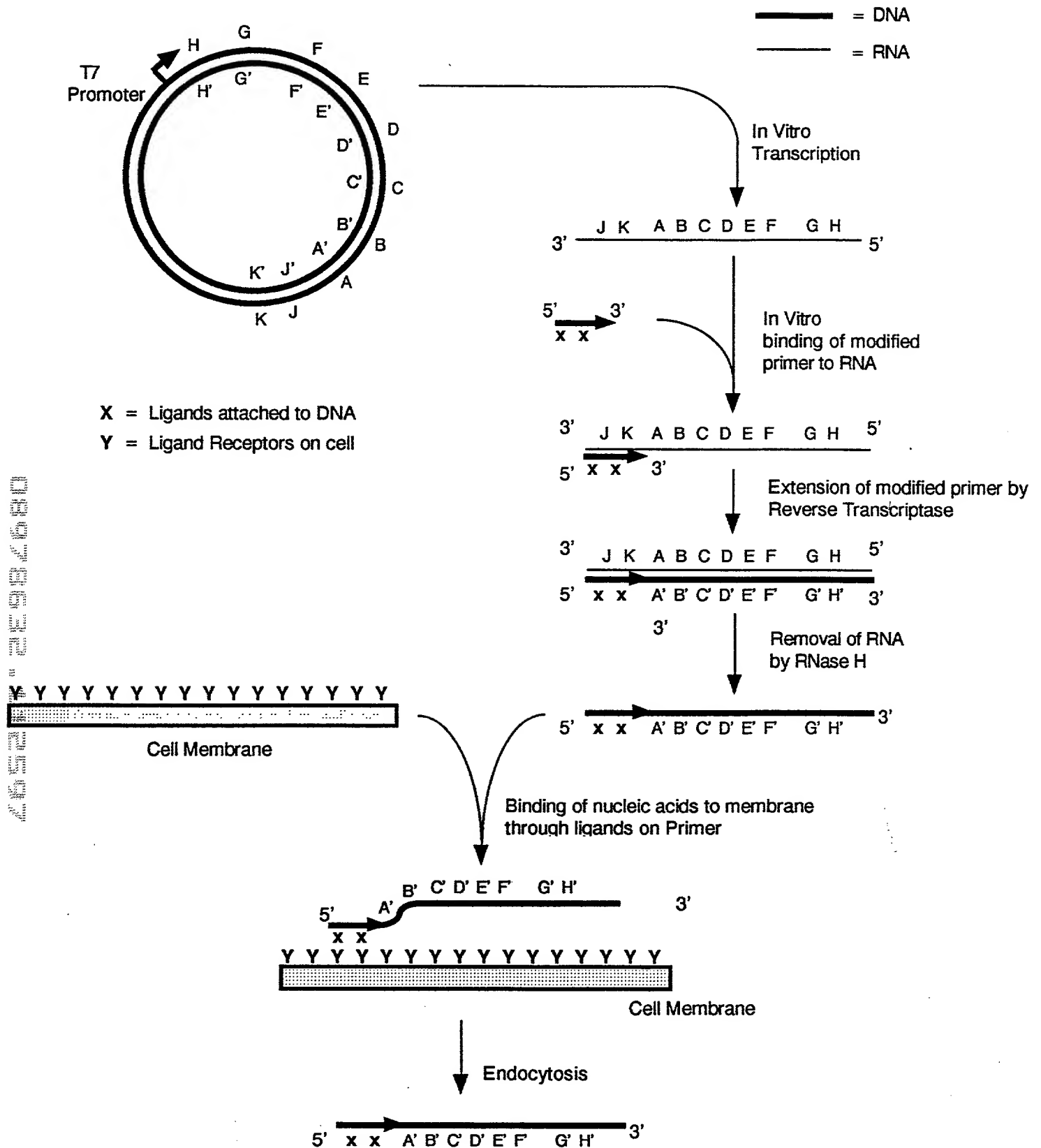
(Continued in Figure 10)

Figure 9
RNA with Ligands on Multiple Primers

Continued from Figure 9

**Figure 10**

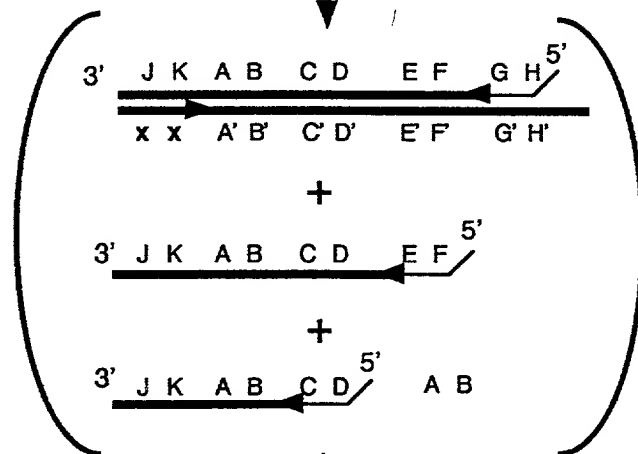
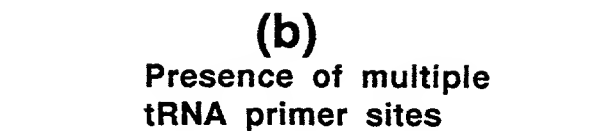
RNA with Ligands on Multiple Primers (Continued)



(Continued in Figure 12)

Figure 11
Single-stranded DNA with attached Ligands

(a)
Presence of a single
tRNA primer site



The diagram illustrates the formation of a double Holliday junction (dHJ) from a double-strand break (DSB). The top part shows a DSB with 3' and 5' ends, and segments labeled J, K, A, B, C, D, E, F, G, H. The bottom part shows the dHJ structure with two DNA molecules, each with segments J, K, A, B, C, D, E, F, G, H, and a central region labeled K' A' B' C' D'.

Figure 12
Single-stranded DNA with attached Ligands (continued)

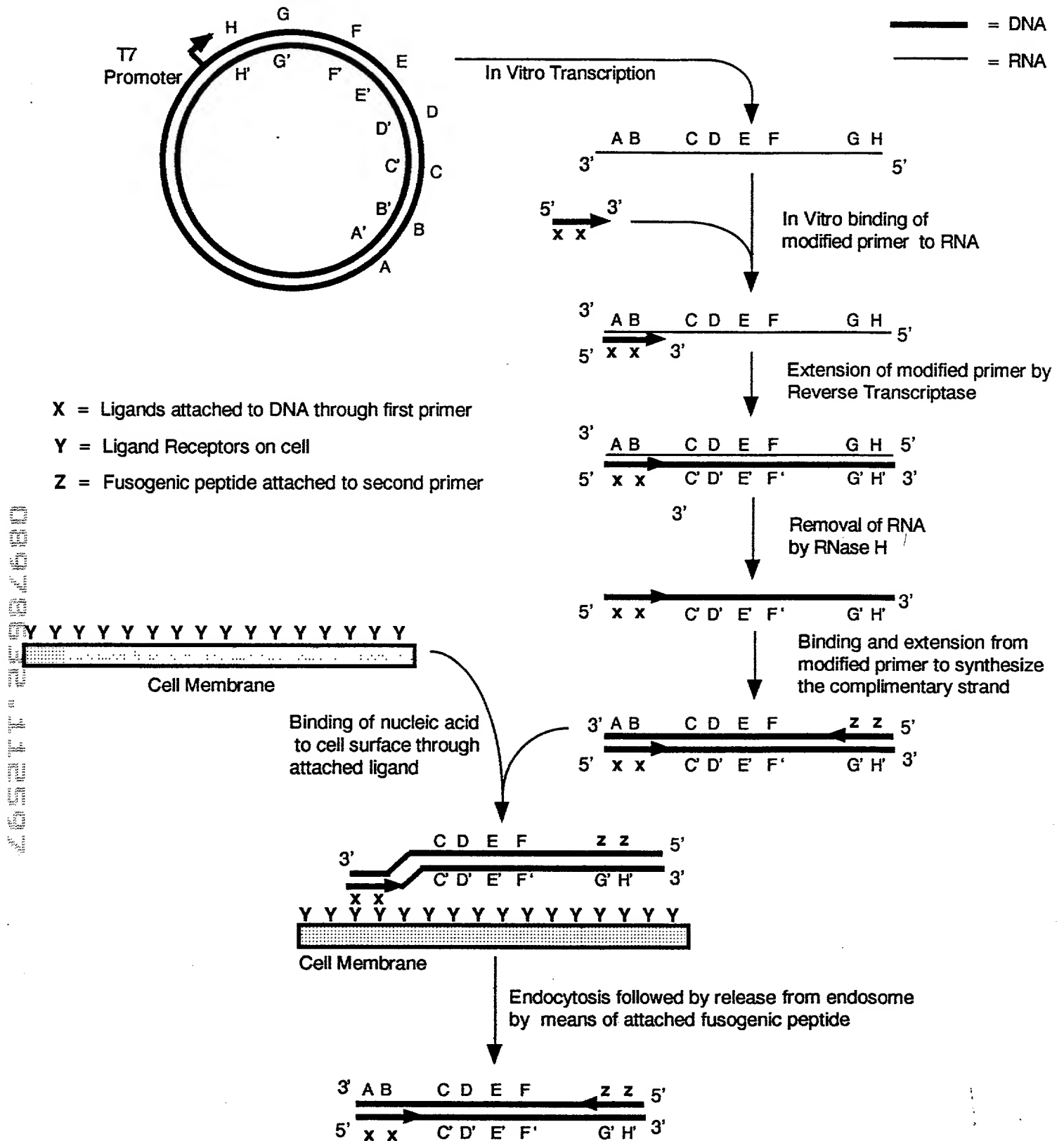


Figure 13

Linear Double-stranded DNA with attached Moieties on each strand

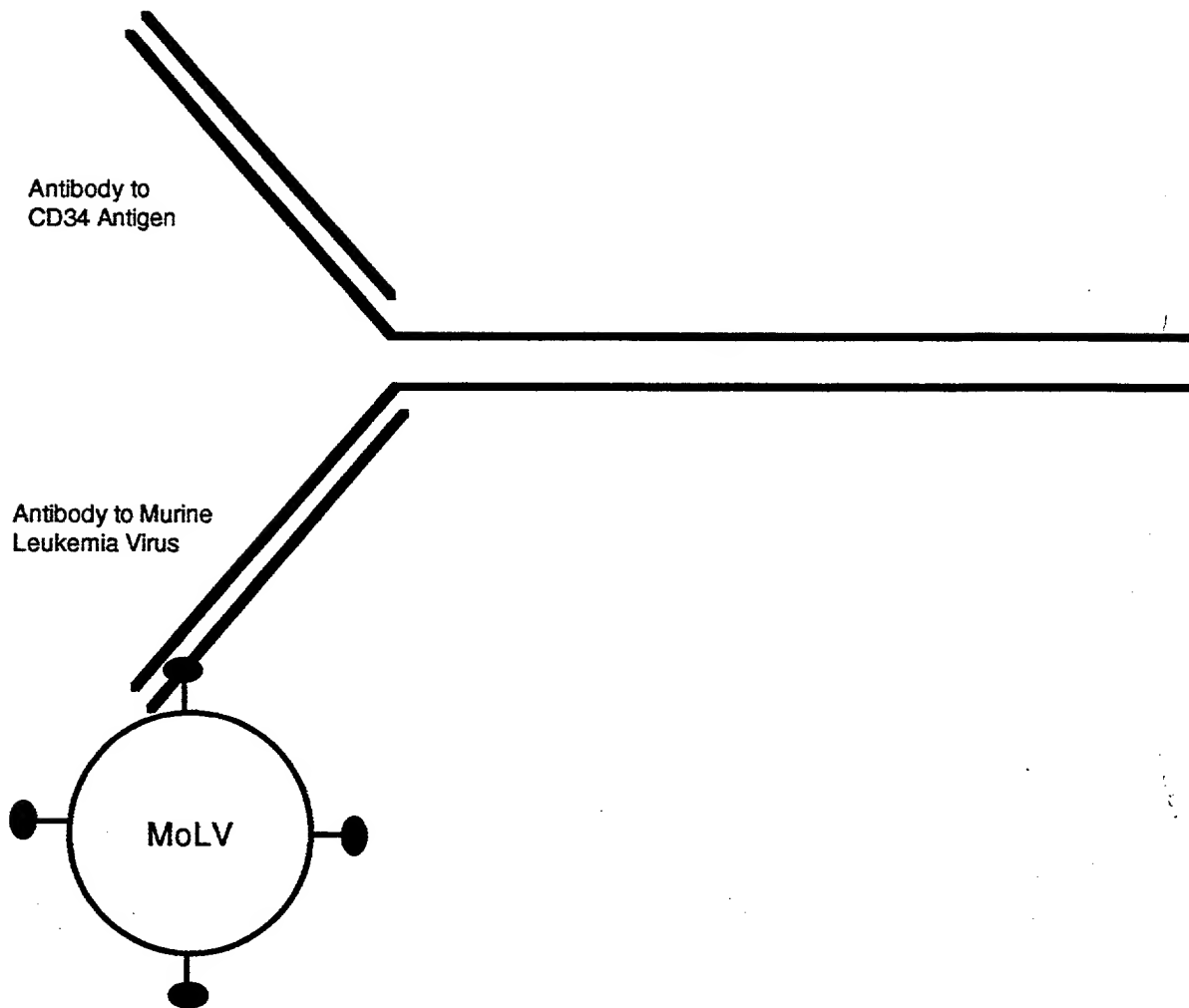


Figure 14

Enhanced Delivery of Retroviral Vector
to Haematopoietic Stem Cell

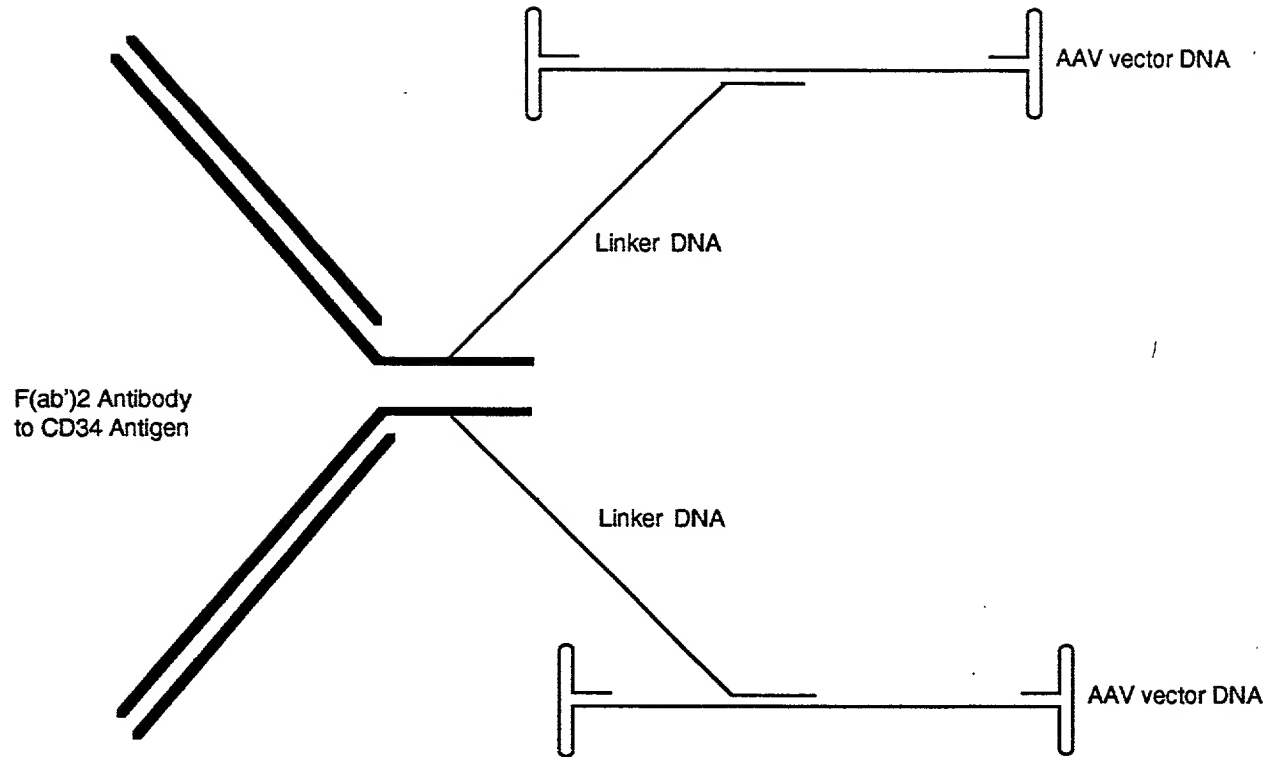


Figure 15

Enhanced Delivery of Vector
DNA to Haematopoietic Stem Cell

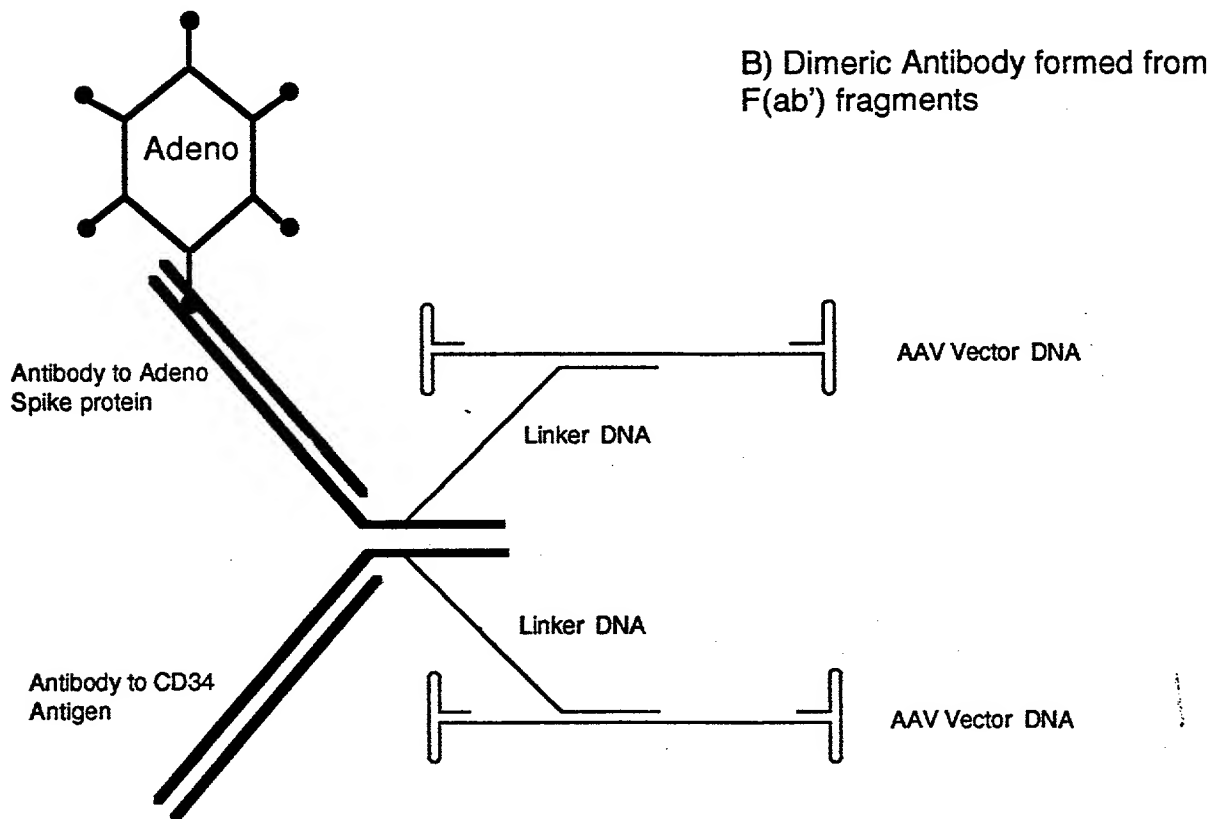
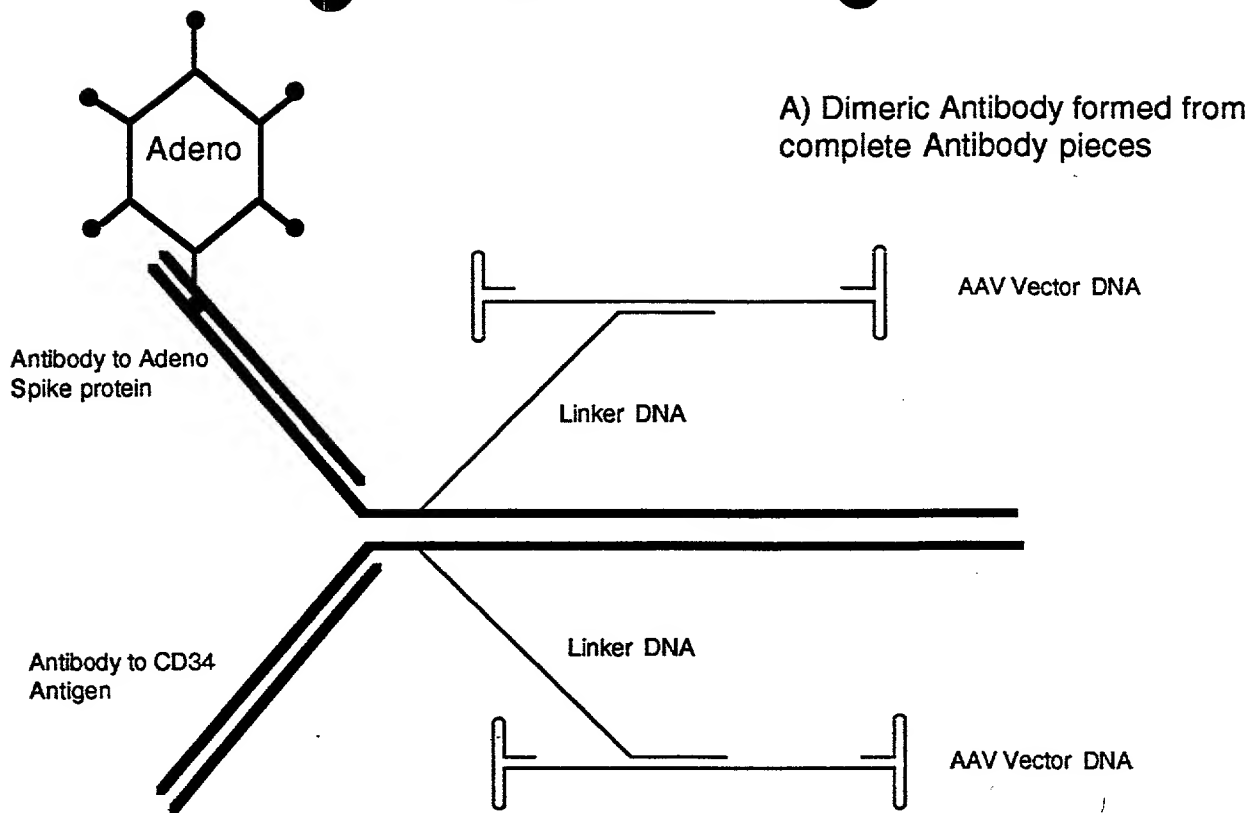


Figure 16
Covalent Attachment of vector DNA to Dimeric Antibody

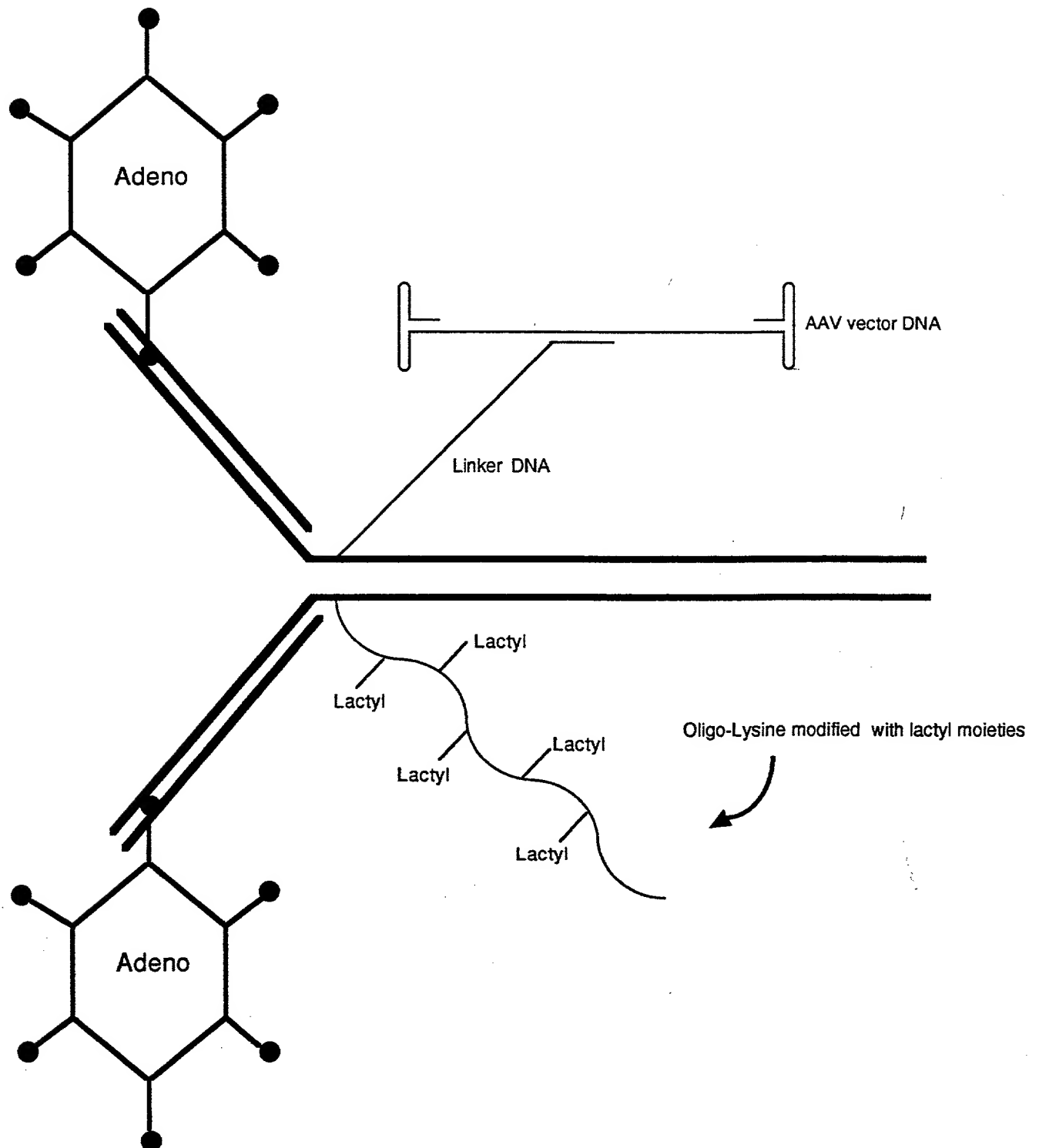


Figure 17
Covalent attachment of Modified DNA
to a Monovalent Antibody

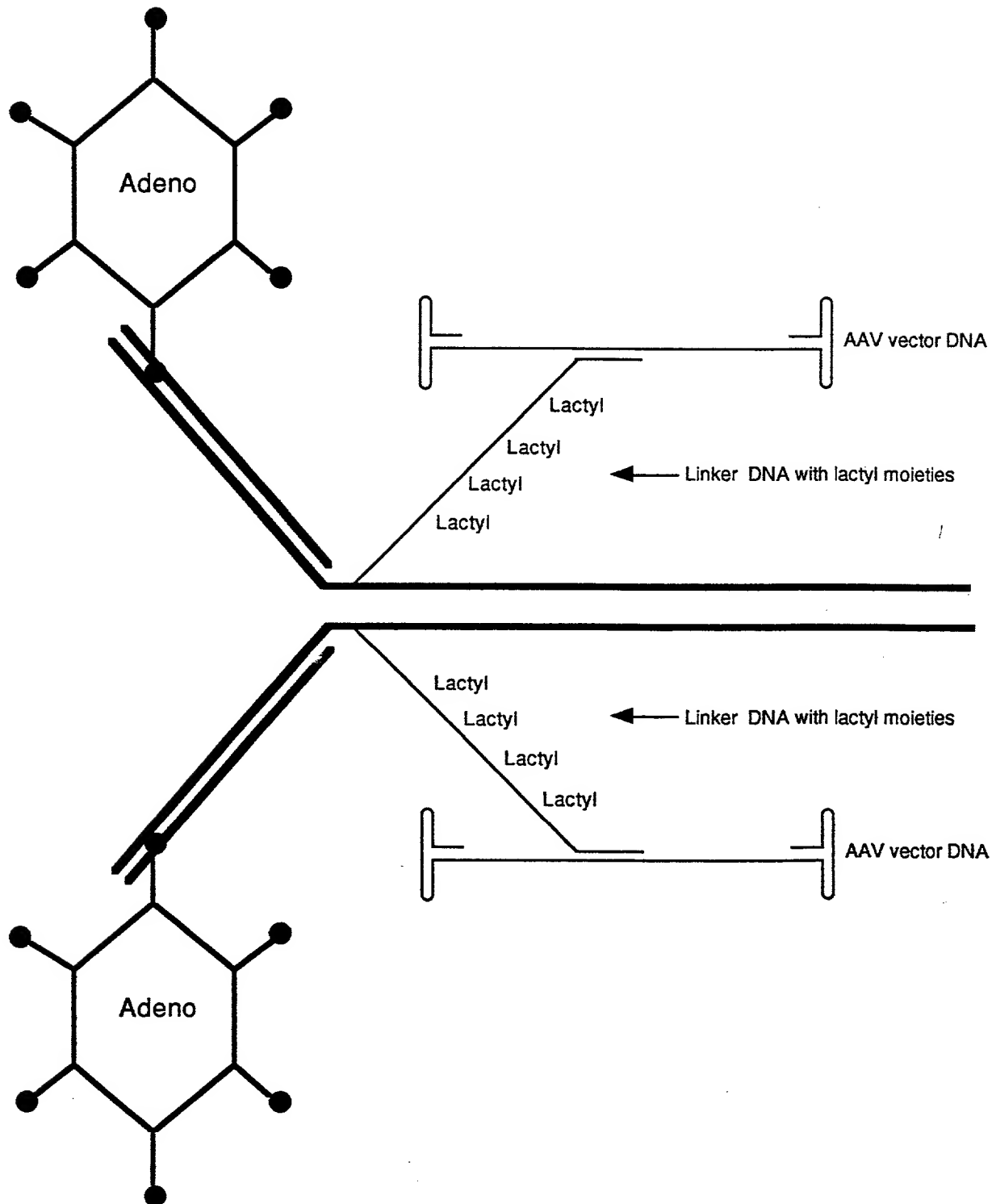
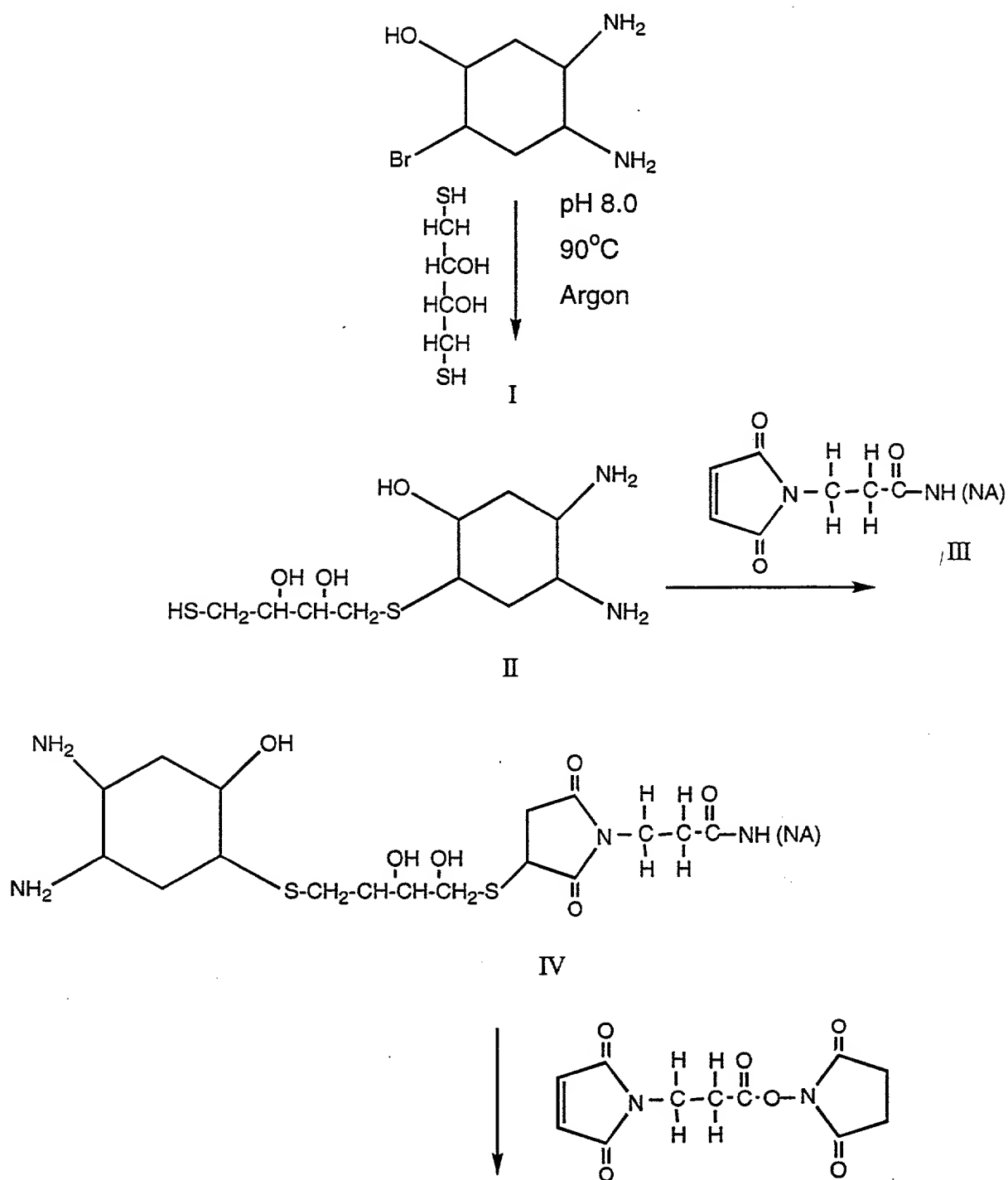


Figure 18
Modified DNA used as a Binder



(continued in Figure 20)

Figure 19
Synthetic Steps for Creation of Antibodies
With Nucleic Acid Moieties Attached

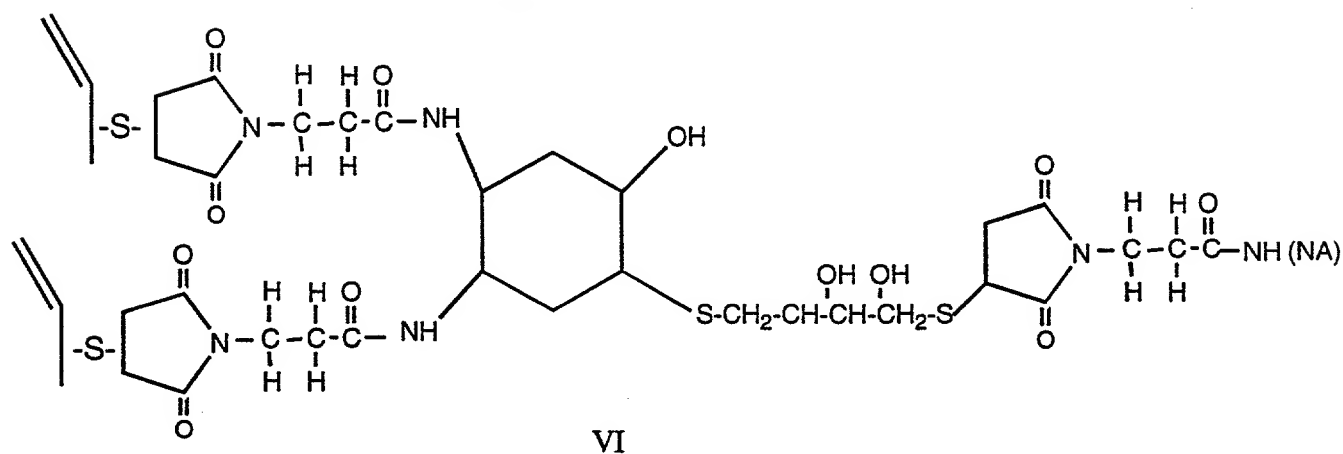
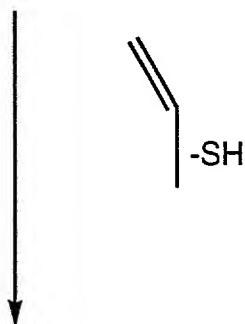
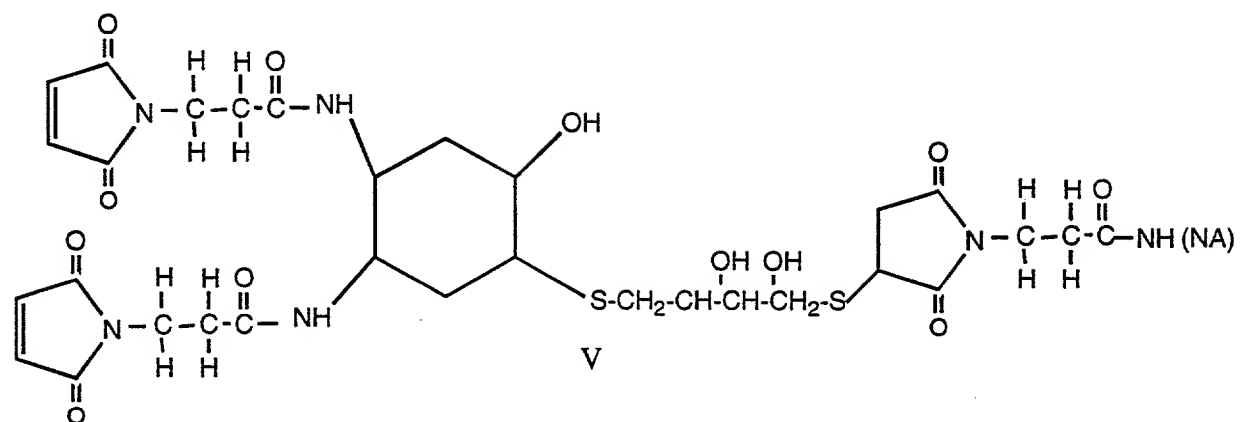


Figure 20
Continuation of Synthetic Steps

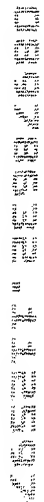


Figure 21

Enhanced Binding of Antibodies to Antigens by Multimerization

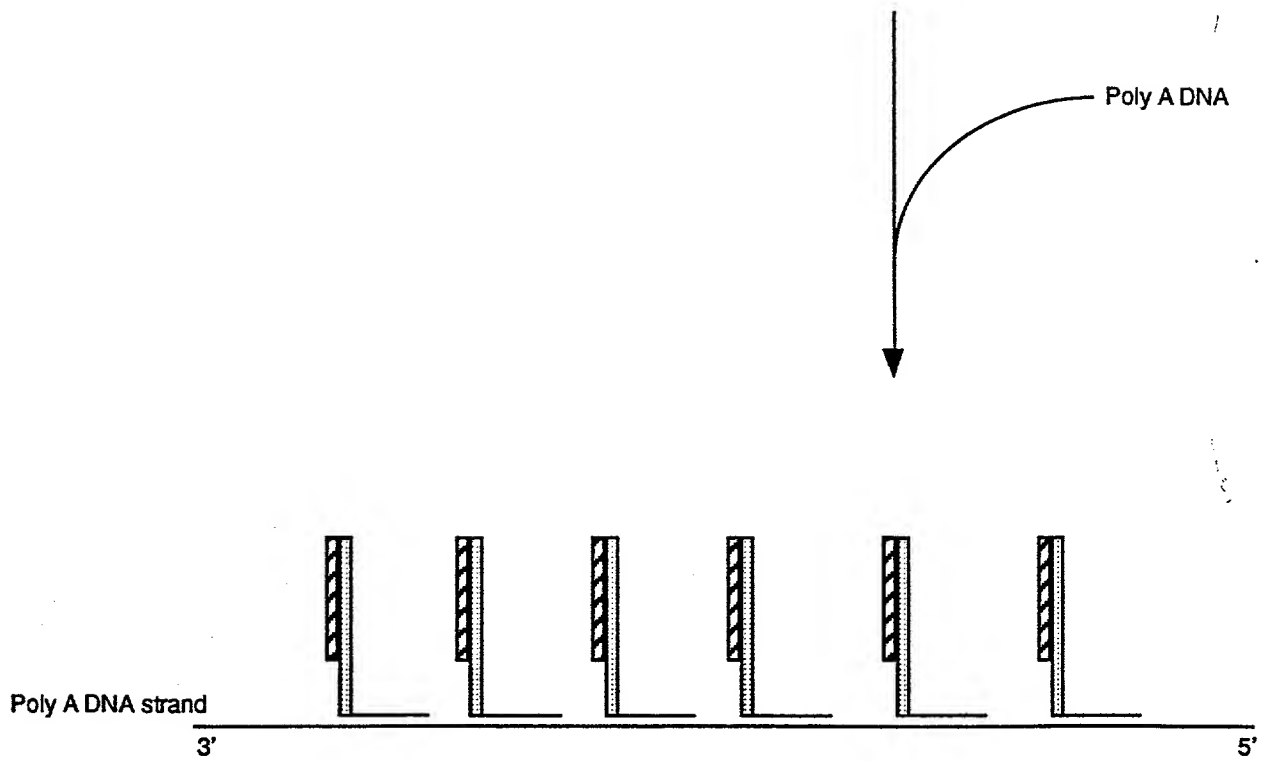
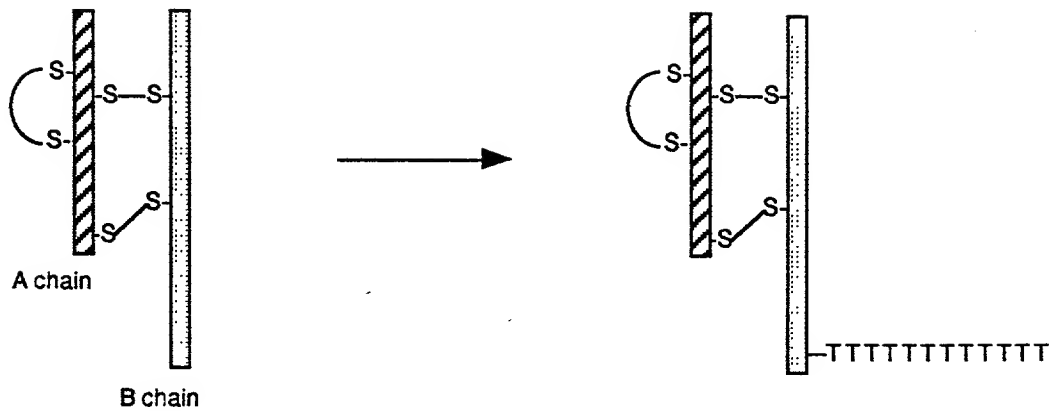


Figure 22
High Affinity Multi-Insulin Soluble Complex

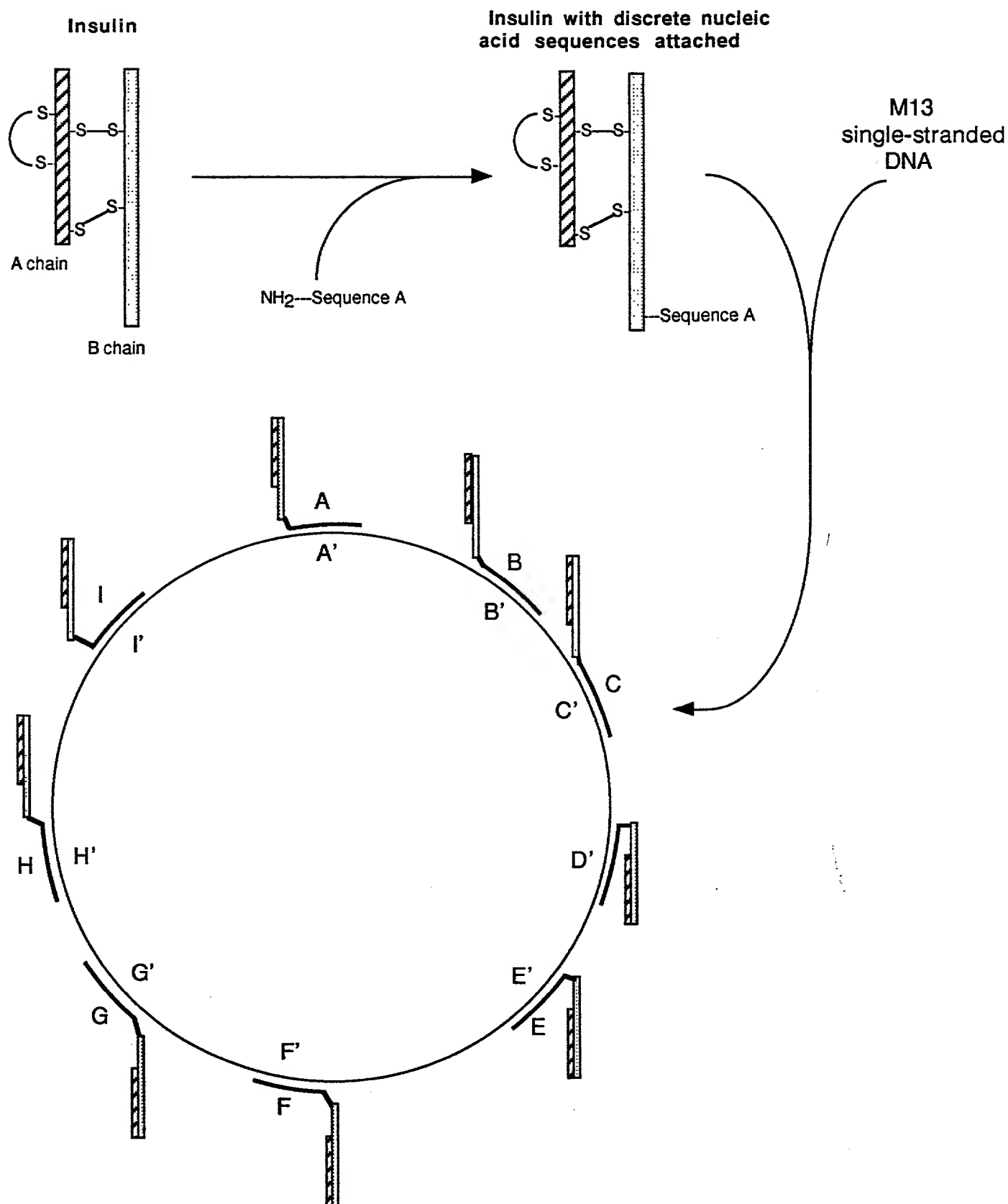


Figure 23

Multimerization of Insulin molecules by hybridization to discrete Sequences

00976633 1159
265477 22952680

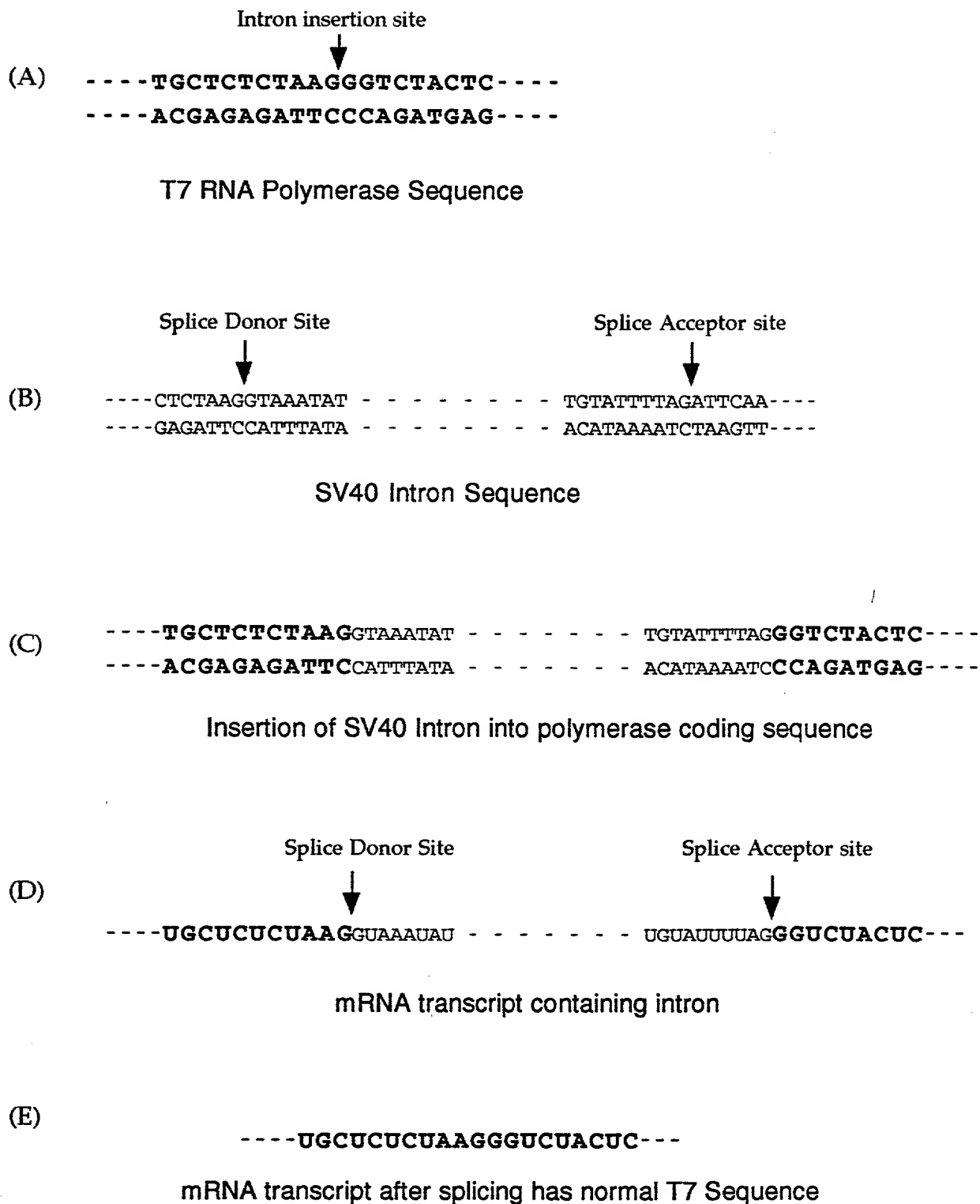
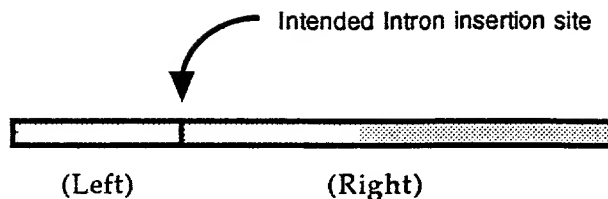


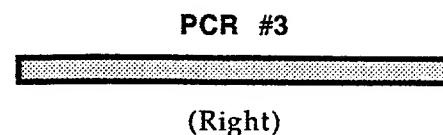
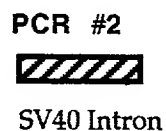
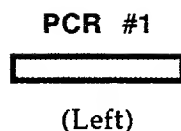
Figure 24

Fusion of Intron into T7 RNA Polymerase Coding Sequence

Normal T7 RNA polymerase coding sequence

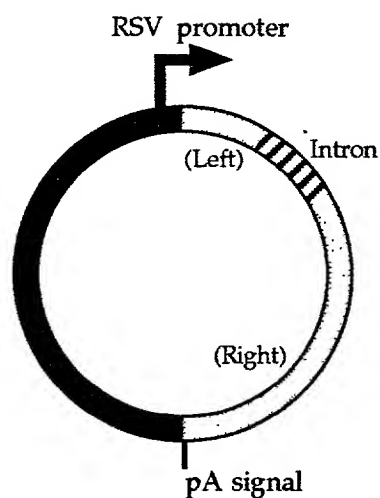


Synthesis of fragments by PCR Amplification of T7 or SV40 templates



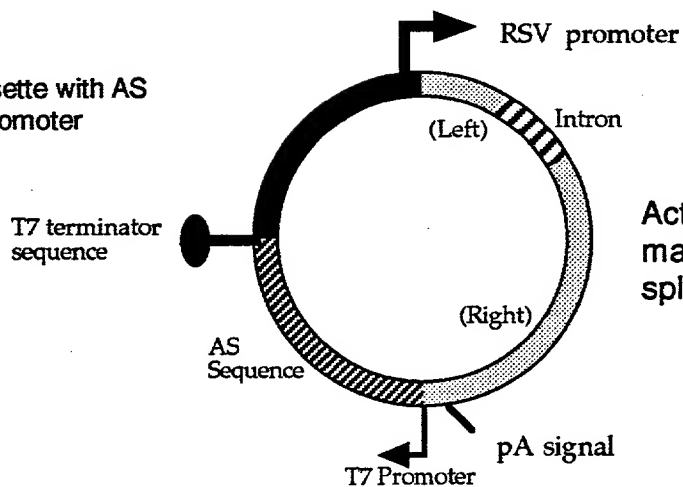
(A)

Fusion of PCR fragments together in eucaryotic expression vector



(B)

Introduction of cassette with AS directed from T7 promoter



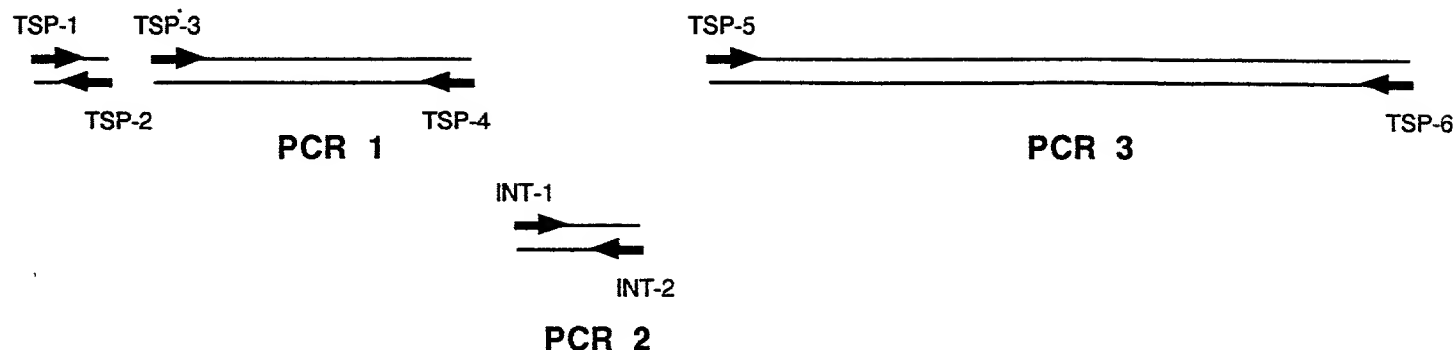
(C)

Active T7 RNA polymerase is only made in eucaryotic cells after splicing out of SV40 Intron

Figure 25

Construction of T7 Expression Vector

A) Synthesis of pieces



B) Oligomers used for synthesis

TSP-1	GGA ATT CGT CTC GAG CTC TGA TCA CCA CCA TGG ACA CGA TTA ACA TCG C
TSP-2	GAC TAG TTG GTC TCG TCT CTT TTT TGG AGG AGT GTC GTT CTT AGC GAT GTT AAT C
TSP-3	GGA ATT CGT CTC GGA GAA AGG TAA AAT TCT CTG ACA TCG AAC TGG C
TSP-4	GAC TAG TGG TCT CCC CTT AGA GAG CAT GTC AGC
TSP-5	GGA ATT CGG TCT CGG GTC TAC TCG GTG GCG AGG
TSP-6	GAC TAG TCG TTA CGC GAA CGC AAA GTC
INT-1	GGA ATT CGT CTC TAA GGT AAA TAT AAA ATT TTT AAG
INT-2	GAC TAG TCG TCT CTG ACC CTA AAA TAC ACA AAC AAT TAG A

Figure 26

Synthesis of Pieces for Construction of
T7 RNA Polymerase with Intron

Formation of Nuclear Localisation Signal by Fusion of TSP1/TSP2 Product to Clone with PCR #1 product

Annealing of TSP1 with TSP2

TSP1

5' GG AAT TCG TCT CGA GCT CTG ATC ACC ATG GAC ACG ATT AAC ATC GC 3' TSP2
3' C TAA TTG TAG CGA TTC TTG CTG TGA GGA GGT TTT TTC TCT GCT CTG GAT CAG 5'

Extension of TSP1/TSP2 by polymerase

5' GG AAT TCG TCT CGA GCT CTG ATC ACC ATG GAC ACG ATT AAC ATC GCT AAG AAC GAC ACT CCT CCA AAA AAG AGA CGA GAC CAA CTA GTC 3'
3' CC TTA AGC AGA GCT CGA GAC GTA TGG TGG TAC CTG TGC TAA TTG TAG CGA TTC TTG CTG TGA GGA GGT TTT TTC TCT GAT CAG 5'

Bsa I

Digestion of TSP1/TSP2 product with Bsa I

5' GG AAT TCG TCT CGA GCT CTG ATC ACC ATG GAC ACG ATT AAC ATC GCT AAG AAC GAC ACT CCT CCA AAA AA
3' CC TTA AGC AGA GCT CGA GAC GTA TGG TGG TAC CTG TGC TAA TTG TAG CGA TTC TTG CTG TGA GGA GGT TTT TTC TCT

Digestion of PCR #1 clone (pL-1) with BsmB I

Bsm BI
5' GGA ATT CGT CTC G GAGA AAG GTA AAA TTC TCT GAC ATC GAA CTG GC-----
CCT TAA GCA GAG CCTCT TTC CAT TTT AAG AGA CTG TAG CTT GAC CG-----

Ligation of Bsa I digested TSP1/TS2 product to BsmB I digested PCR#1 clone

5' GG AAT TCG TCT CGA GCT CTG ATC ACC ATG GAC ACG ATT AAC ATC GCT AAG AAC GAC ACT CCT CCA AAA AAG AGA AAG GTA AAA TTC
3' CC TTA AGC AGA GCT CGA GAC GTA TGG TGG TAC CTG TGC TAA TTG TAG CGA TTC TTG CTG TGA GGA GGT TTT TTC TCT TTC CAT TTT AAG
TCT GAC ATC GAA CTG GC-----
AGA CTG TAG CTT GAC CG-----

Figure 27

Comparison of the 5' ends of the Nucleotide Sequences of Wild Type and Modified T7 RNA Polymerase

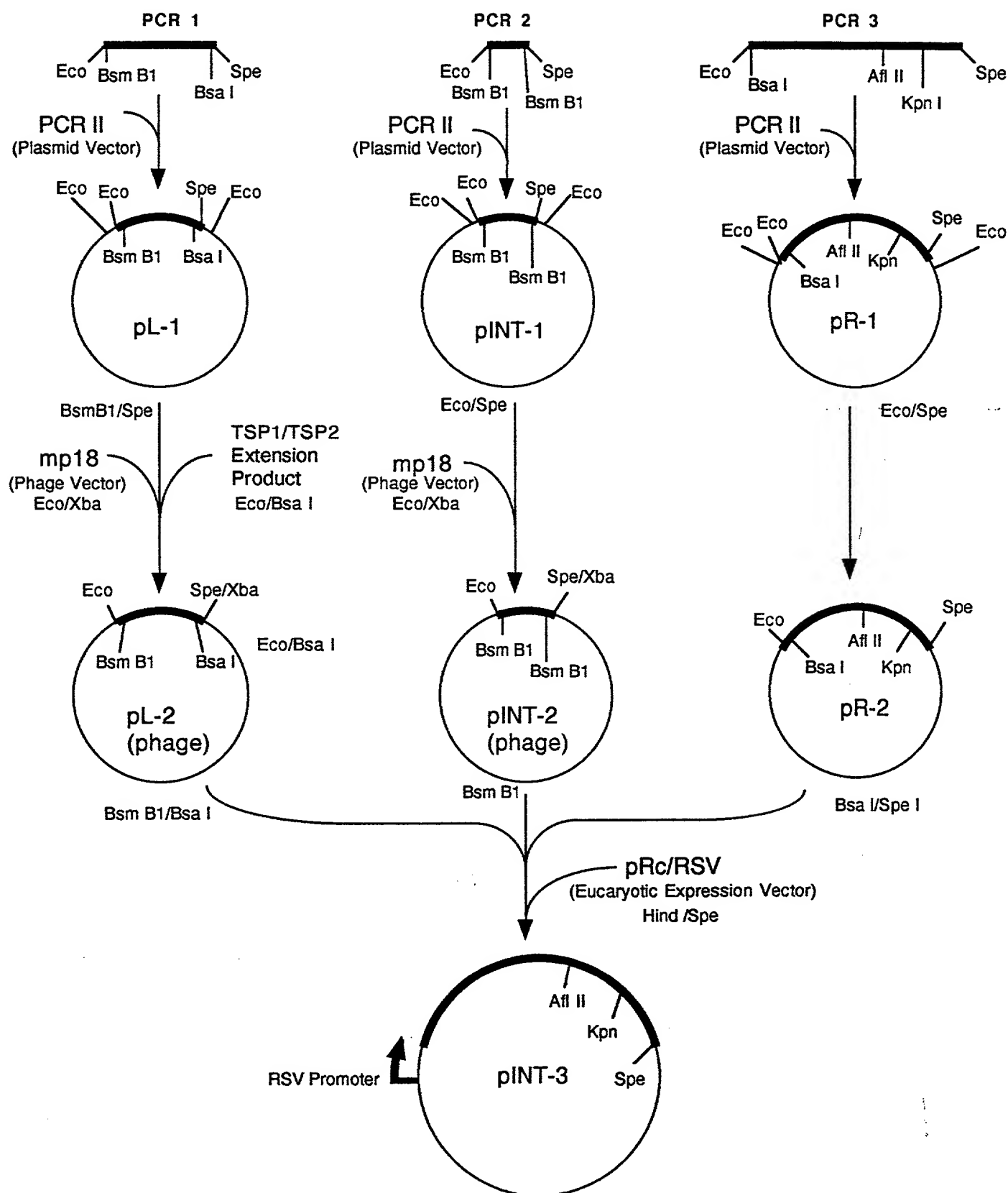
Wild Type T7 nucleic and amino acid sequence

ATG	GAC	ACG	ATT	AAC	ATC	GCT	AAG	AAC	GAC	TTC	TCT	GAC	ATC	GAA	CTG	GC-----
TAC	CTG	TGC	TAA	TTG	TAG	CGA	TTG	CTG	AAG	AGA	CTG	TAG	CTT	GAC	CG-----	
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	

Modified T7 nucleic and amino acid sequence with Nuclear Localisation Signal (NLS) insertion

ATG	GAC	ACG	ATT	AAC	ATC	GCT	AAG	AAC	GAC	ACT	CCT	CCA	AAA	AAG	AGA	AAG	GTA	AAA	TTC	TCT	GAC	ATC	GAA	CTG	GC-----	
TAC	CTG	TGC	TAA	TTG	TAG	CGA	TTG	CTG	TTG	TGA	GGA	GGT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	AAG	AGA	CTG	TAG	CTT	GAC	CG-----
1	2	3	4	5	6	7	8	9	10											11	12	13	14	15	16	

Figure 28

**Figure 29**

**Fusion of PCR Pieces to Construct
T7 RNA Polymerase with an Intron**

(A) Oligomers

HTA-1	GAT CAT TAG ACC AGA TCT GAG CCT GGG AGC TCT CTG GCT AAC TAG GGA ACC CAC TGCTTA AGC CTC AAG
HTA-2	GAT CCT TGA GGC TTA AGC AGT GGG TTC CCT AGT TAG CCA GAG AGC TCC CAG GCT CAG ATC TGG TCT AAT
HTB-1	GAT CAC CTT AGG CTC TCC TAT GGC AGG AAG AAG CGG AGA CAG CGA CGA AGA CCT CCT CAA G
HTB-2	GAT CCT TGA GGA GGT CTT CGT CGC TGT CTC CGC TTC TTC CTG CCA TAG GAG AGC CTA AGG T
HTC-1	GAT CAT AGT GAA TAG AGT TAG GCA GGG ATA CTC ACC ATT ATC GTT TCA GAC CCA CCT CCC AG
HTC-2	GAT CCT GGG AGG TGG GTC TGA AAC GAT AAT GGT GAG TAT CCC TGC CTA ACT CTA TTC ACT AT
TER-1	AAT CTA GAG CTA ACA AAG CCC GAA AGG AAG
TER-2	TTC TGC AGA TAT AGT TCC TCC TTT CAG C

(B) Cloning of AS and Terminator sequences into vector with T7 Promoter

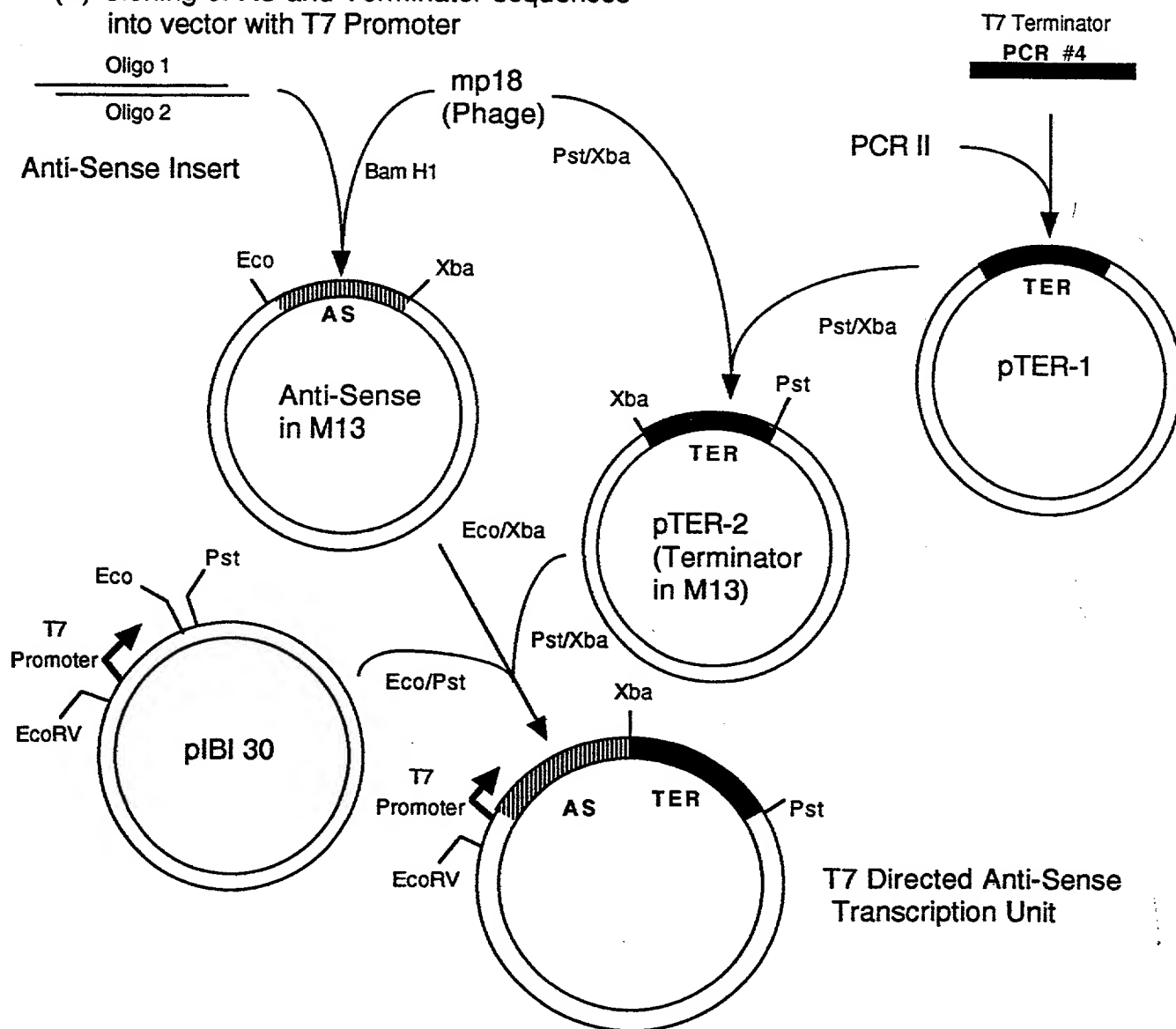


Figure 30
Insertion of Anti-Sense Sequences into
T7 Directed Transcription Units

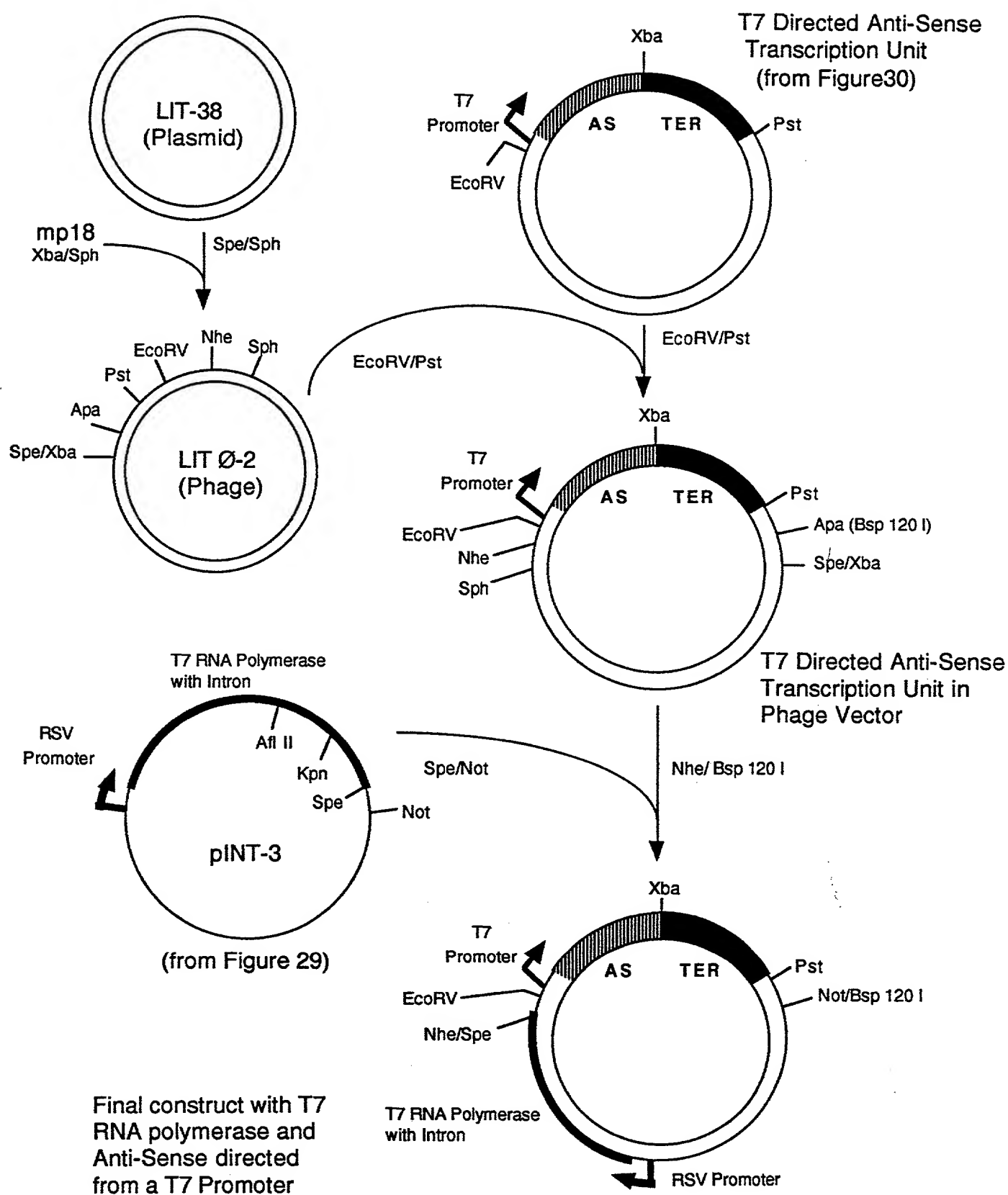


Figure 31

Construct with T7 RNA polymerase and Anti-Sense directed from a T7 Promoter

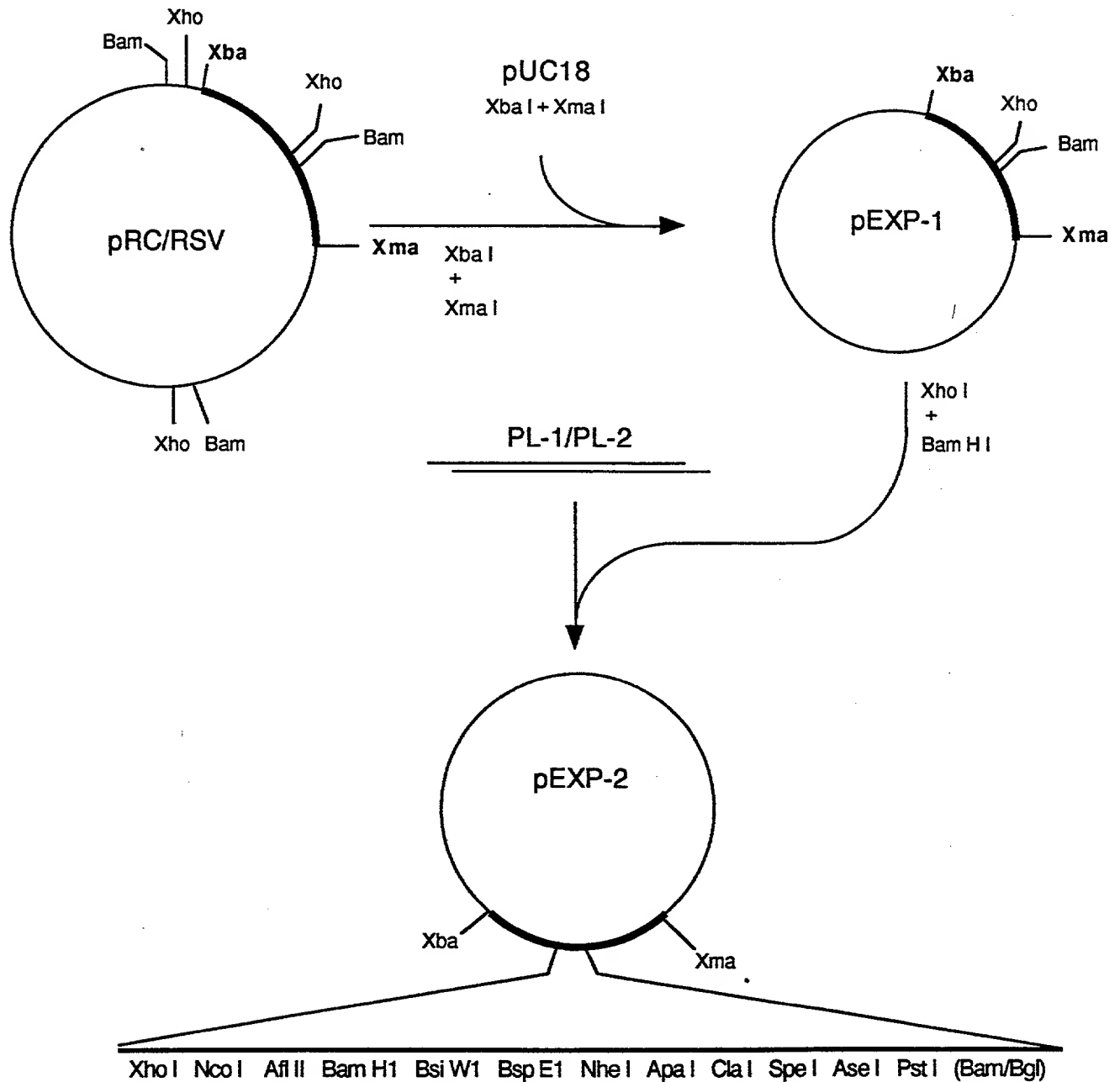
A) Oligomers for introduction of T7 signals and polylinker

PL-1

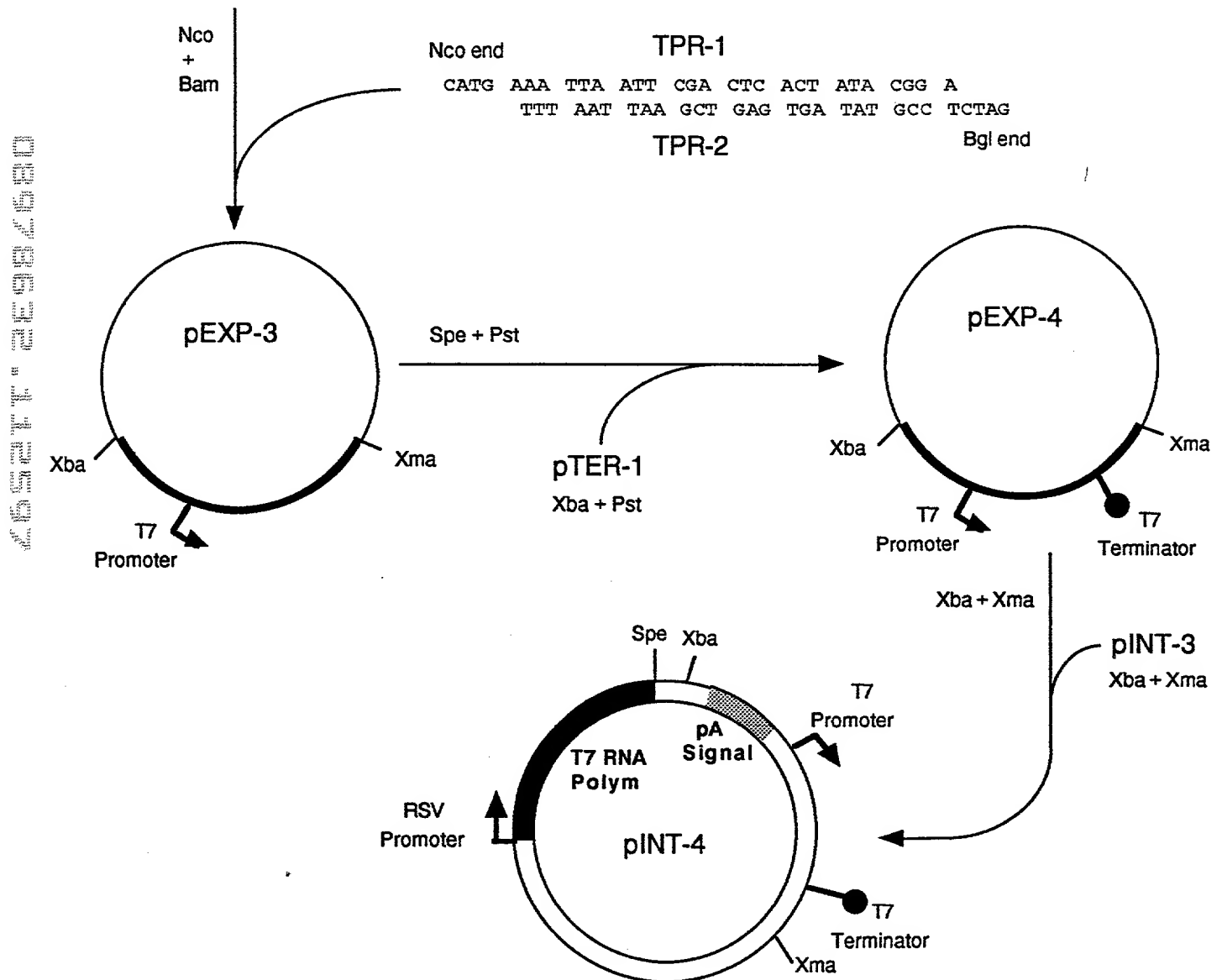
TCG AGC CAT GGC TTA AGG ATC CGT ACG TCC GGA GCT AGC GGG CCC ATC GAT ACT
AGT TAA ATG CAG ATC T

PL-2

CTA GAG ATC TGC ATT TAA CTA GTA TCG ATG GGC CCG CTA GCT CCG GAC GTA CGG
ATC CTT AAG CCA TGG C

**Figure 32**

Introduction of Poly-Linker for Creation of Protein Expression Vector

**Figure 33**

Final steps for construction of Expression Vector

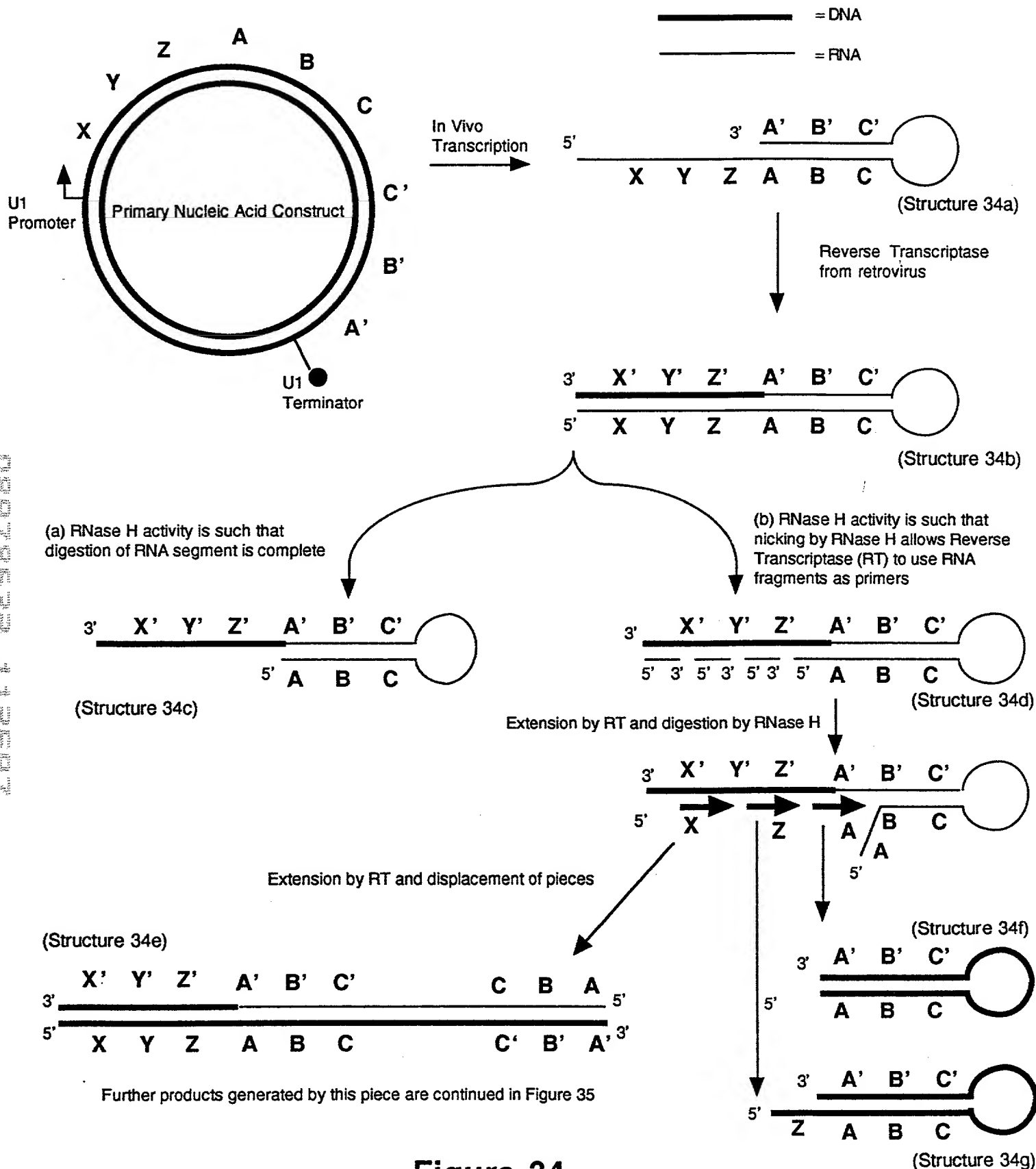
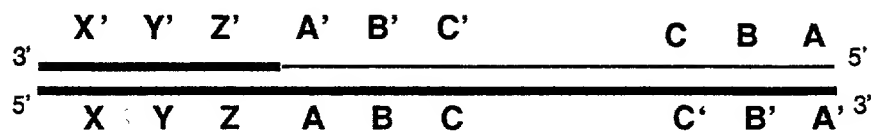


Figure 34

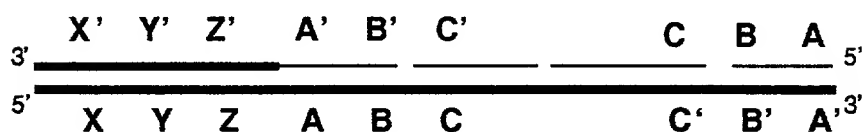
Construct that produces single-stranded Anti-Sense DNA

Continued from Figure 34

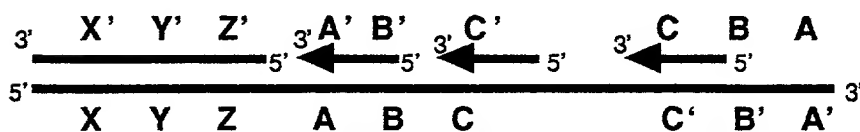
(Structure 34e)



Nicking by RNase H



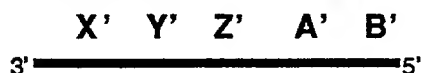
Extension by RT and digestion by RNase H



(Structure 35h)



(Structure 35i)



(Structure 35j)



(Structure 35k)

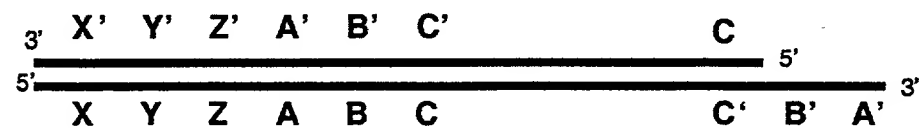
Extension by RT and displacement generates
Single-Stranded DNA and a mostly Double-stranded
DNA molecule

Figure 35
Continuation of Process from Figure 34

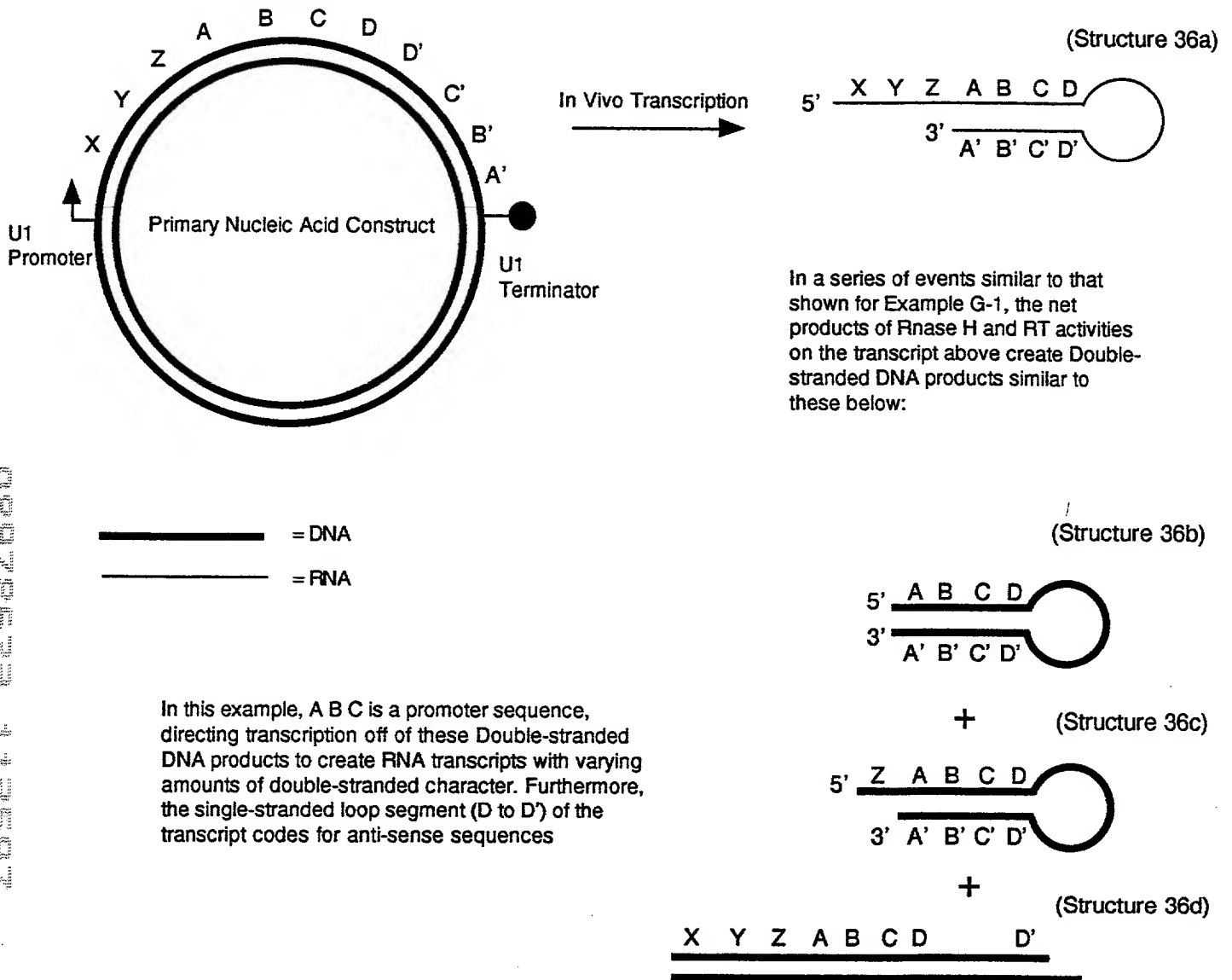
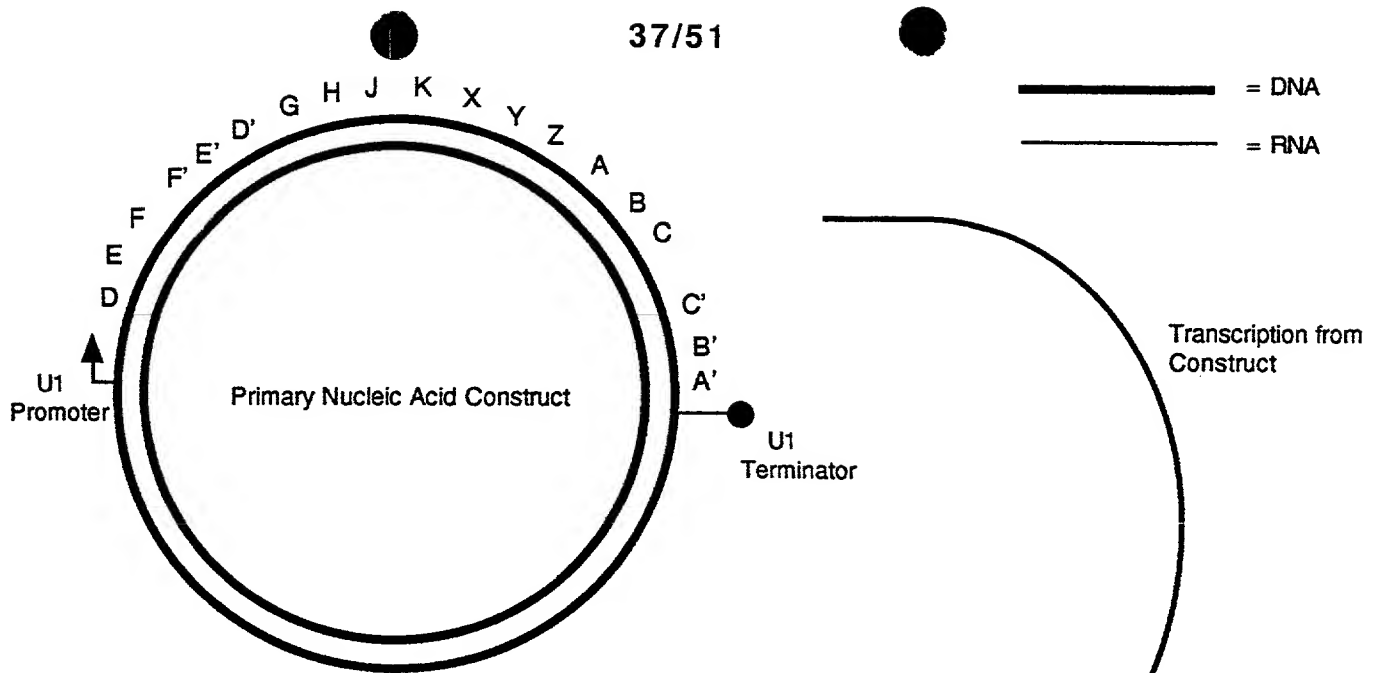
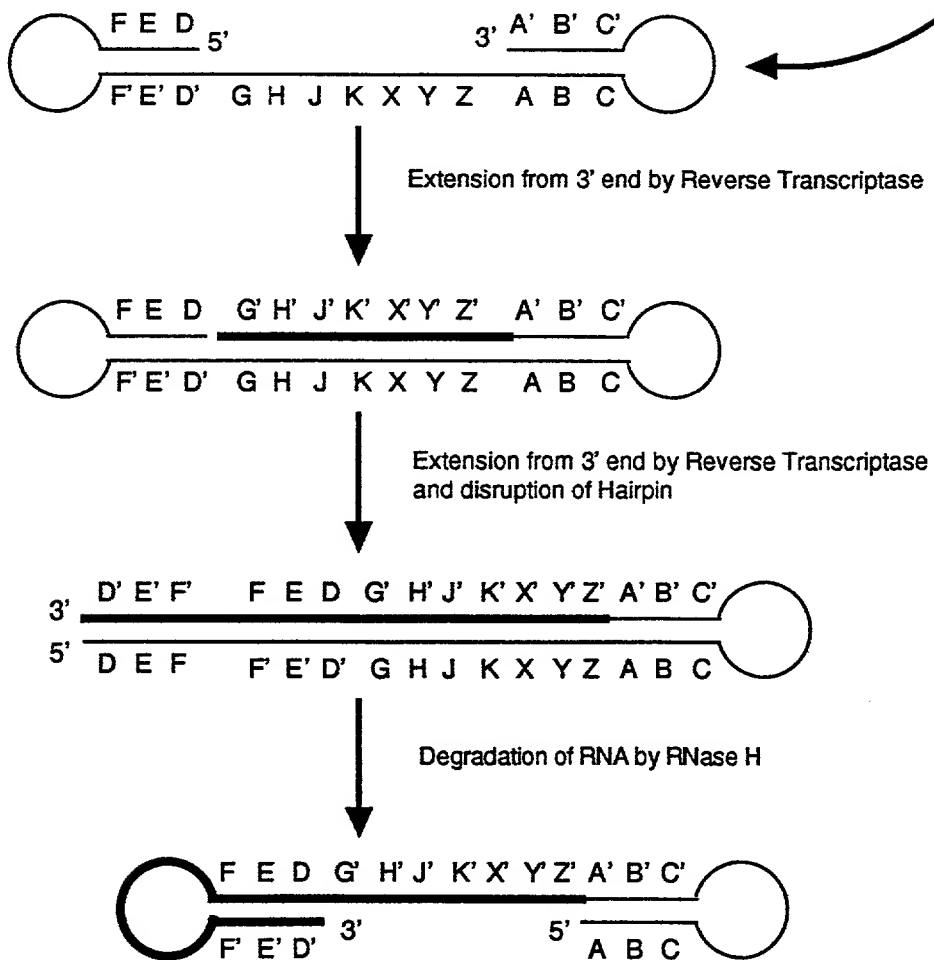


Figure 36

Construct that produces RNA that is Reverse Transcribed to create Secondary DNA Constructs capable of directing transcription



(Structure 37a)



(Continued in Figure 38)

Figure 37

Construct which Propagates a Double Hairpin Production Center

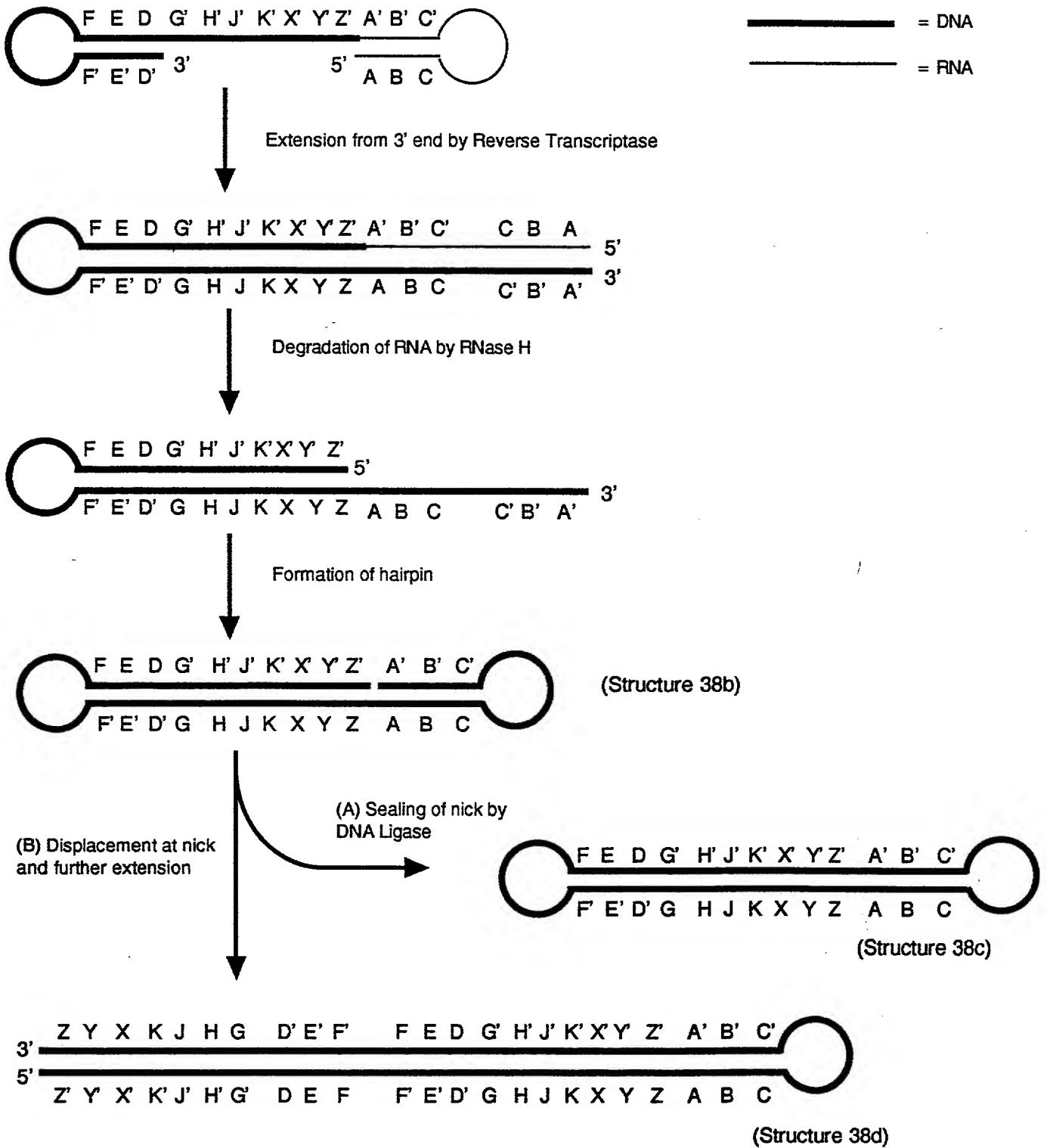
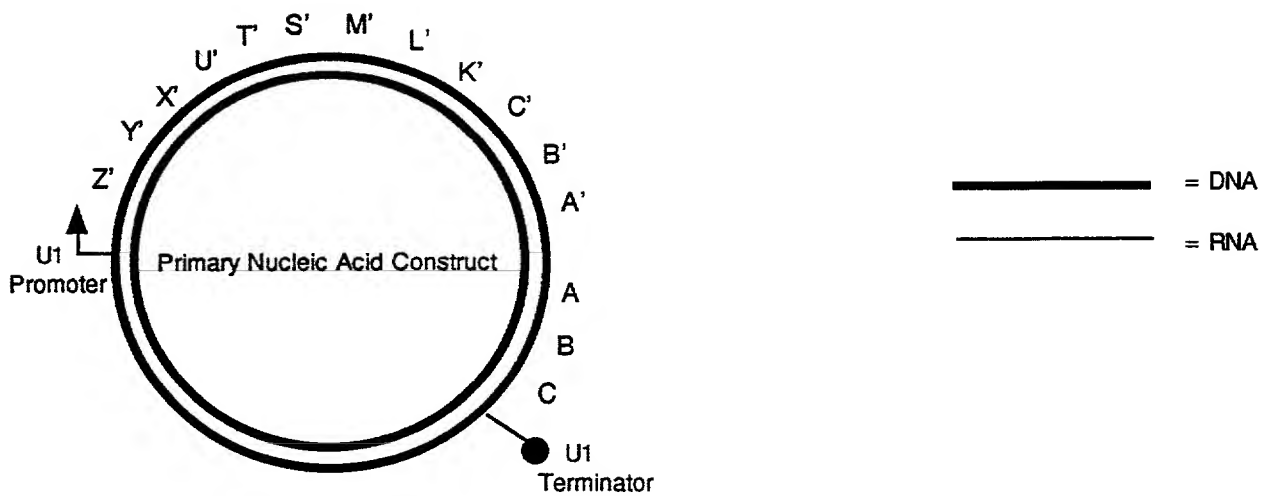
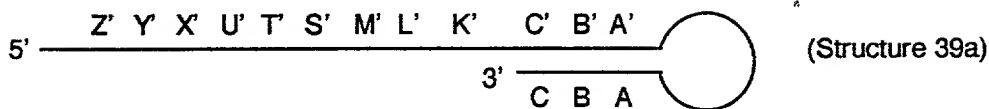


Figure 38

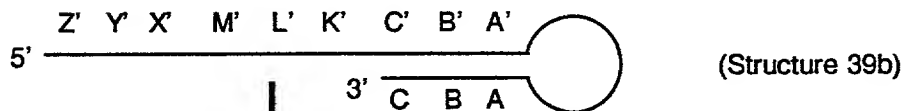
Continuation of process from Figure 37



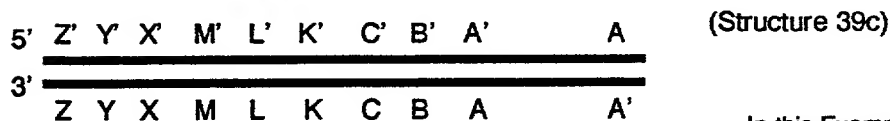
In vivo transcription



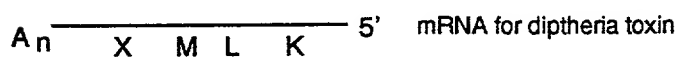
Splicing out of Intron on Anti-coding strand. In this example the sequence X' U' T' S' M' reflects splice donor, intron and splice acceptor sequences.



A series of RNase H and Reverse Transcriptase steps as shown in Figures 34 and 35



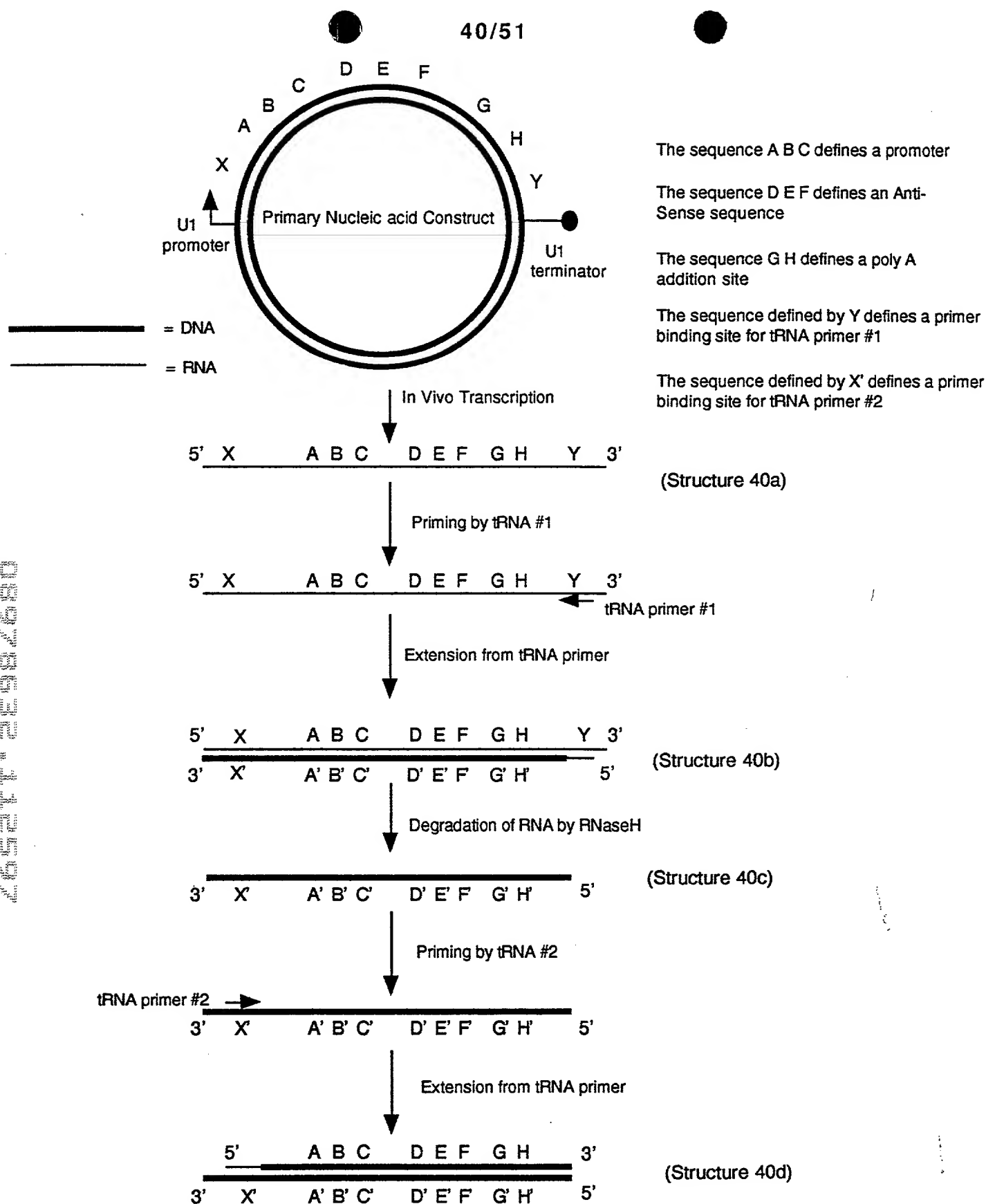
In vivo transcription

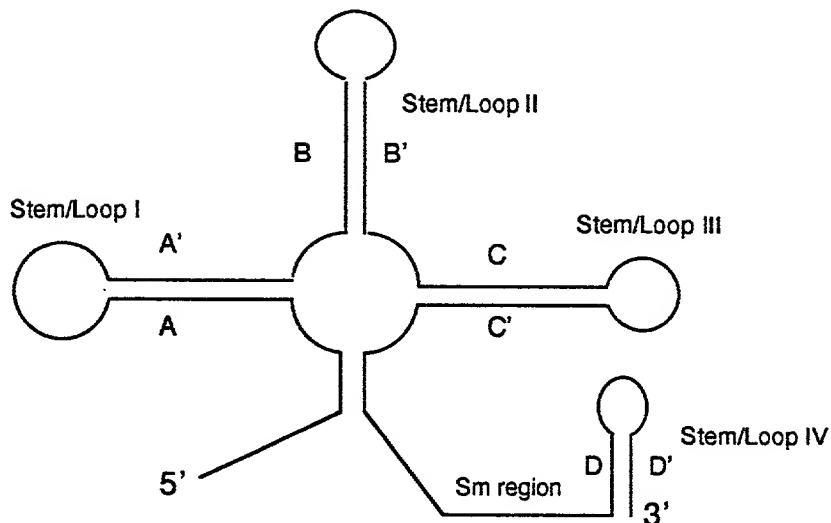


In this Example:
 A B C defines the sequence of an HIV LTR,
 K L M X defines the sequence of diptheria toxin
 YZ defines the sequence for a poly A signal.

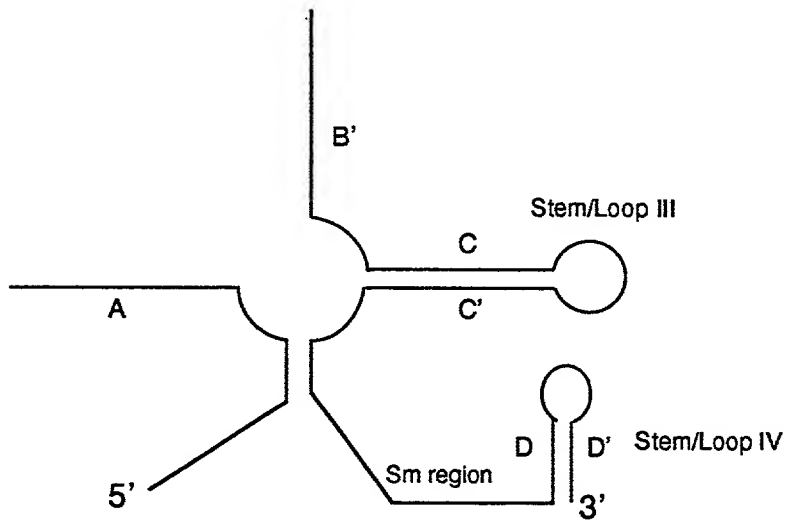
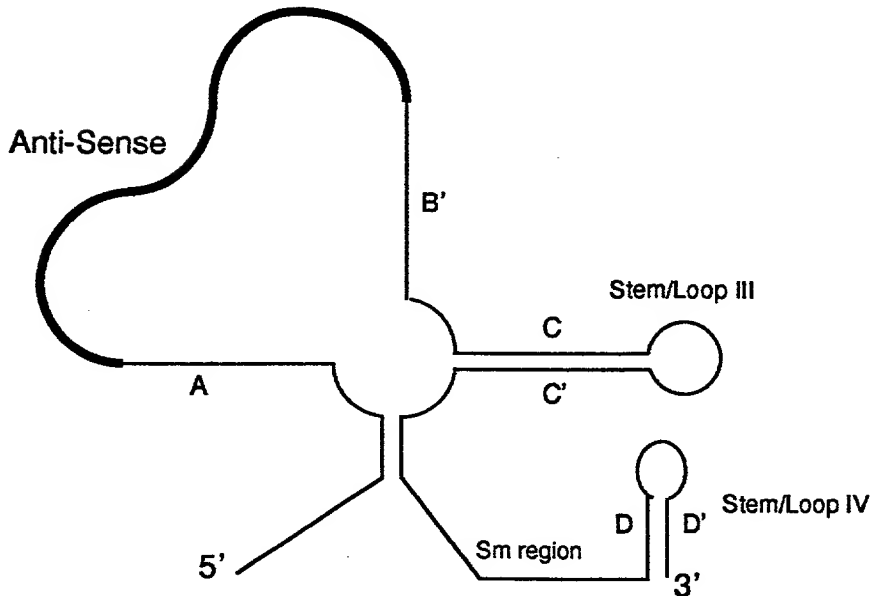
Figure 39

Construct which propagates a Production Center capable of Inducible Suicide





Normal U1

U1 with
Bcl 1/Bsp EI
piece removedU1 with Anti-Sense
sequence inserted**Figure 41**

Excision of Sequences from U1 Transcript Region
and Replacement with Novel Sequences

(A) Anti-sense oligomers

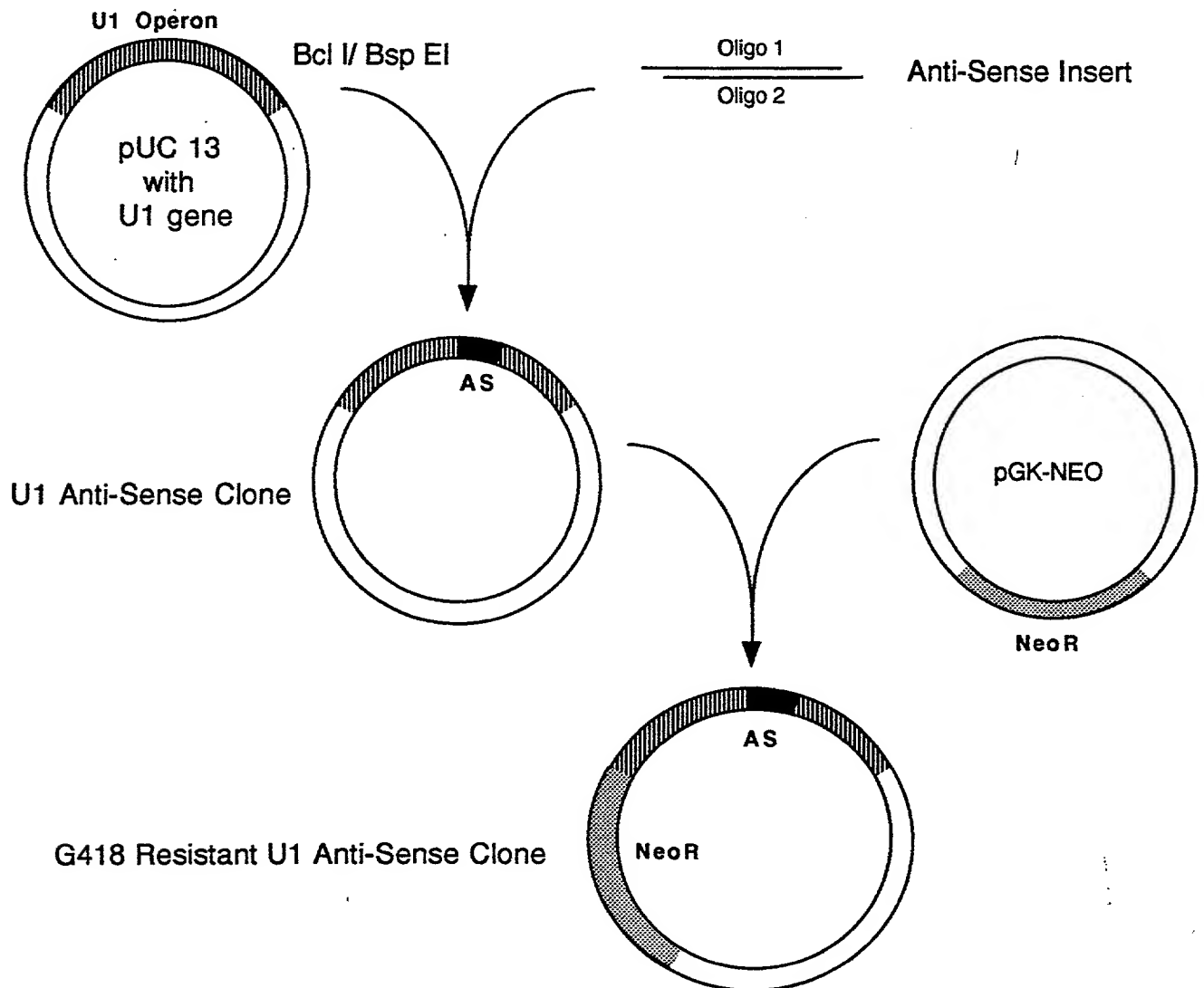
HVA-1 GAT CCG GAT TGA GGC TTA AGC AGT GGG TTC CCT AGT TAG CCA GAG AGC TCC CAG GCT CAG ATC TGG TCT AAT
HVA-2 CCG GAT TAG ACC AGA TCT GAG CCT GGG AGC TCT CTG GCT AAC TAG GGA ACC CAC TGC TTA AGC CTC AAT CCG

HVB-1 GAT CCG GAC CTT GAG GAG GTC TTC GTC GCT GTC TCC GCT TCT TCC TGC CAT AGG AGA GCC TAA GGT
HVB-2 CCG GAC CTT AGG CTC TCC TAT GGC AGG AAG AAG CGG AGA CAG CGA CGA AGA CCT CCT CAA GGT CCG

HVC-1 GAT CCG GAT GGG AGG TGG GTC TGA AAC GAT AAT GGT GAG TAT CCC TGC CTA ACT CTA TTC ACT AT
HVC-2 CCG GAT AGT GAA TAG AGT TAG GCA GGG ATA CTC ACC ATT ATC GTT TCA GAC CCA CCT CCC ATC CG

HVD-1 GAT CAG CAT GCC TGC AGG TCG ACT CTA GAC CCG GGT ACC GAG CTC GCC CTA TAG TGA GT C GTA TTA T
HVD-2 CCG GAT AAT ACG ACT CAC TAT AGG GCG AGC TCG GTA CCC GGG TCT AGA GTC GAC CTG CAG GCA TGC T

(B) Replacement of U1 sequences with HIV Anti-sense sequences

**Figure 42**

Insertion of Anti-Sense Sequences into U1 Operons

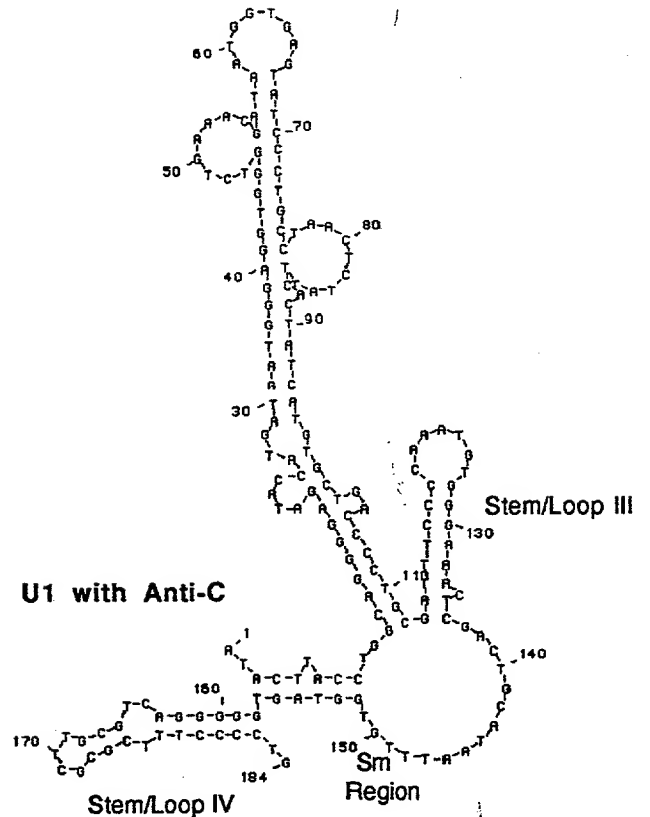
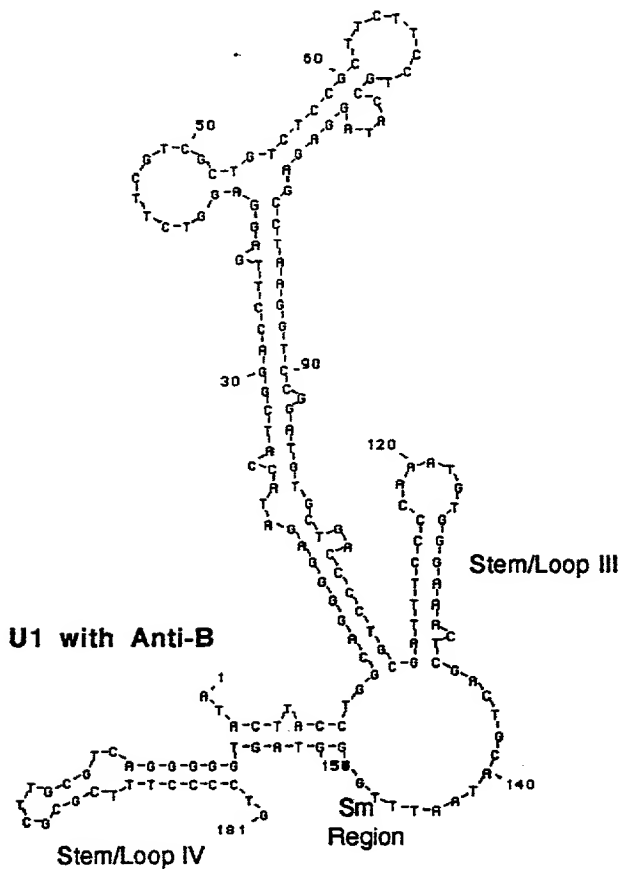
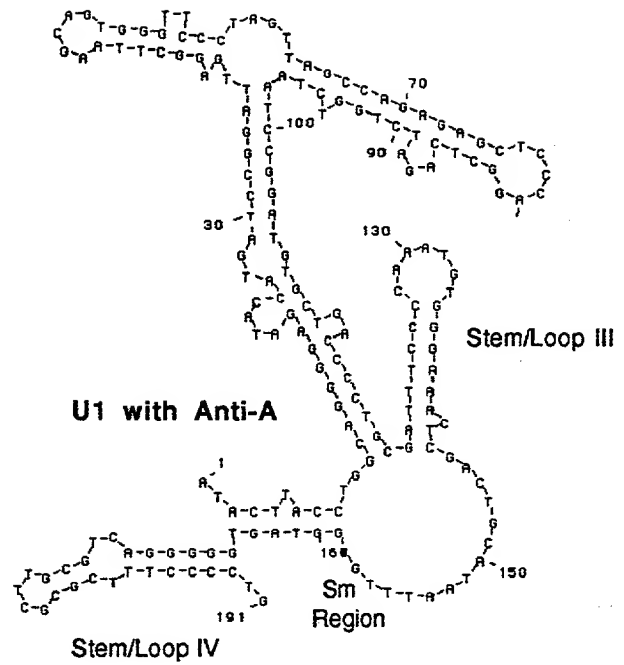
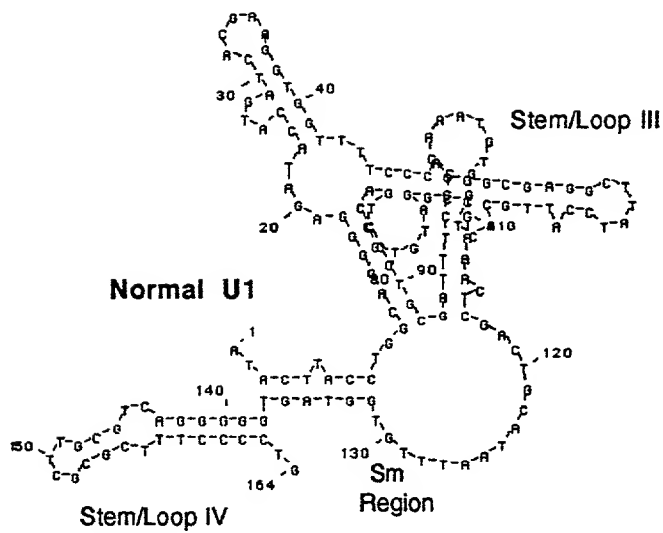


Figure 43

**Predicted Secondary structures for U1
Transcripts with Anti-sense Substitutions**

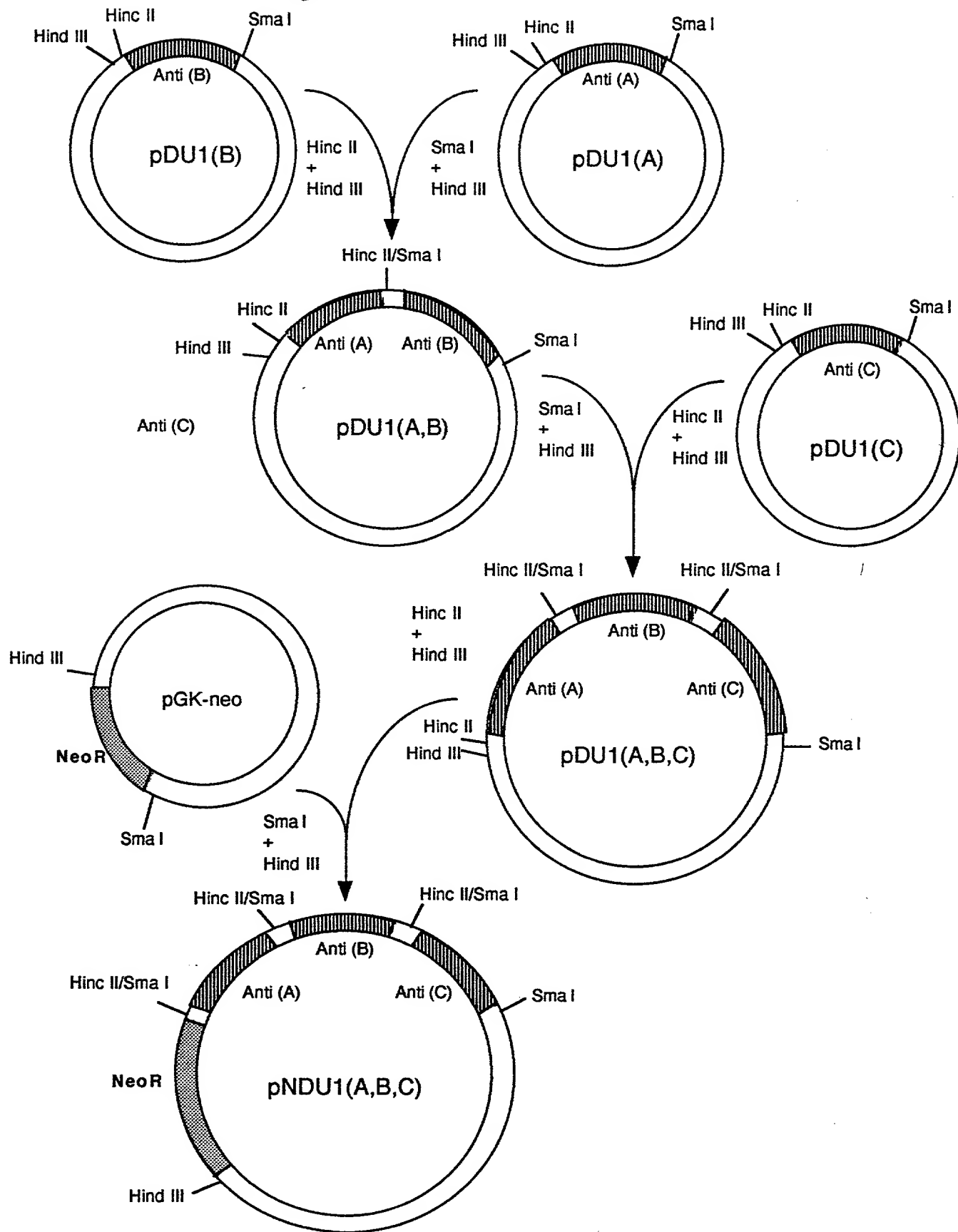


Figure 44
Construction of U1 Multiple Operon Clone

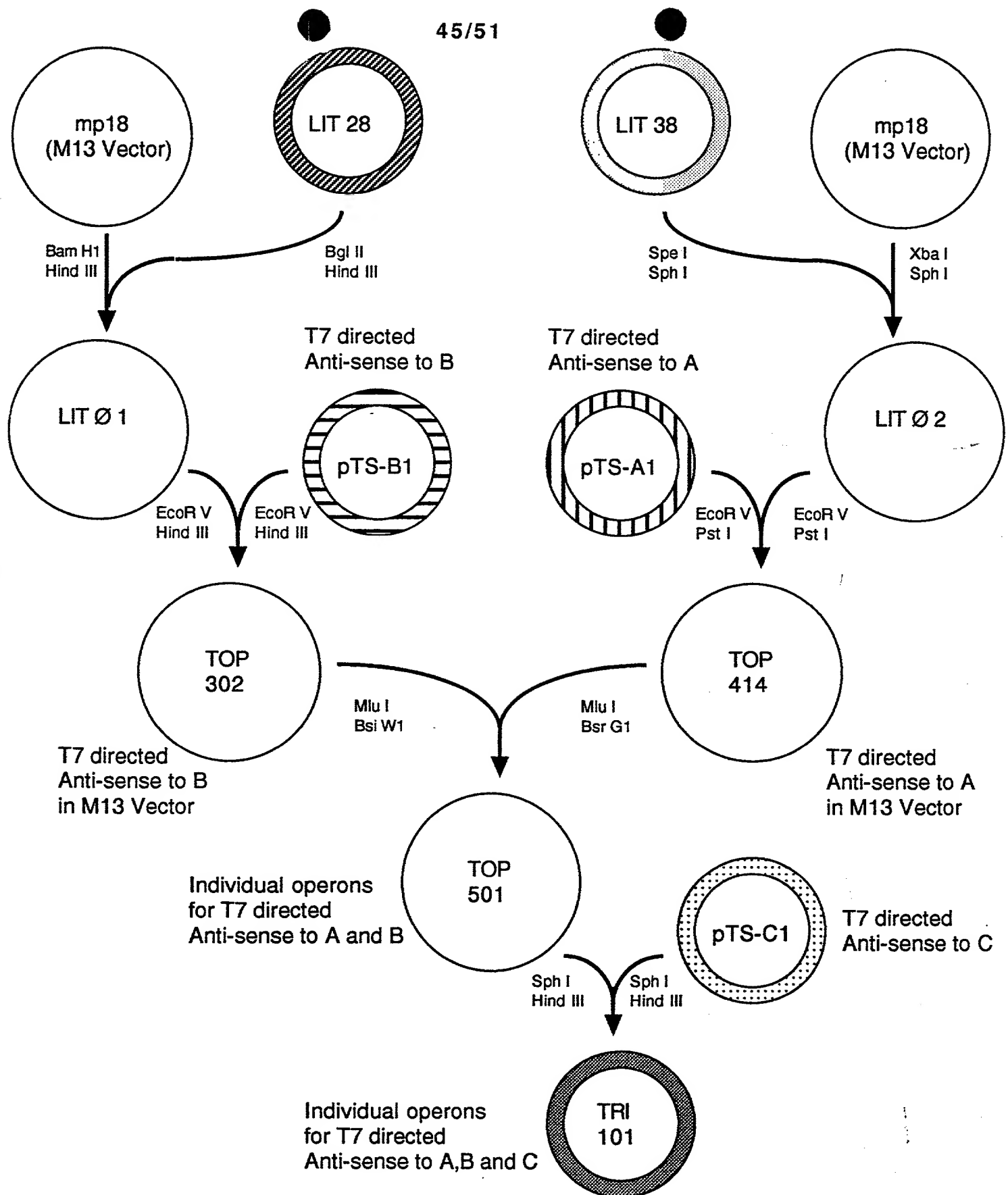
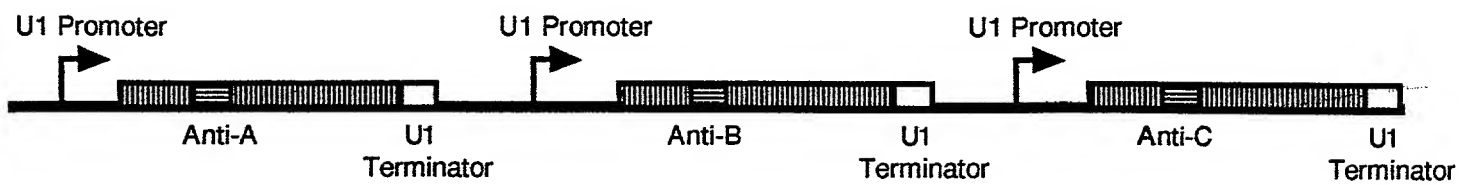


Figure 45
Construction of T7 Triple Operon

pNDU1(A,B,C)

Triple U1 Operon Construct with HIV Anti-Sense



TRI 101

Triple T7 Operon Construct with HIV Anti-Sense

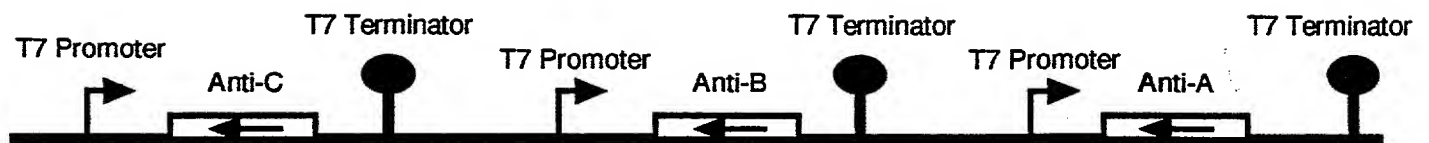
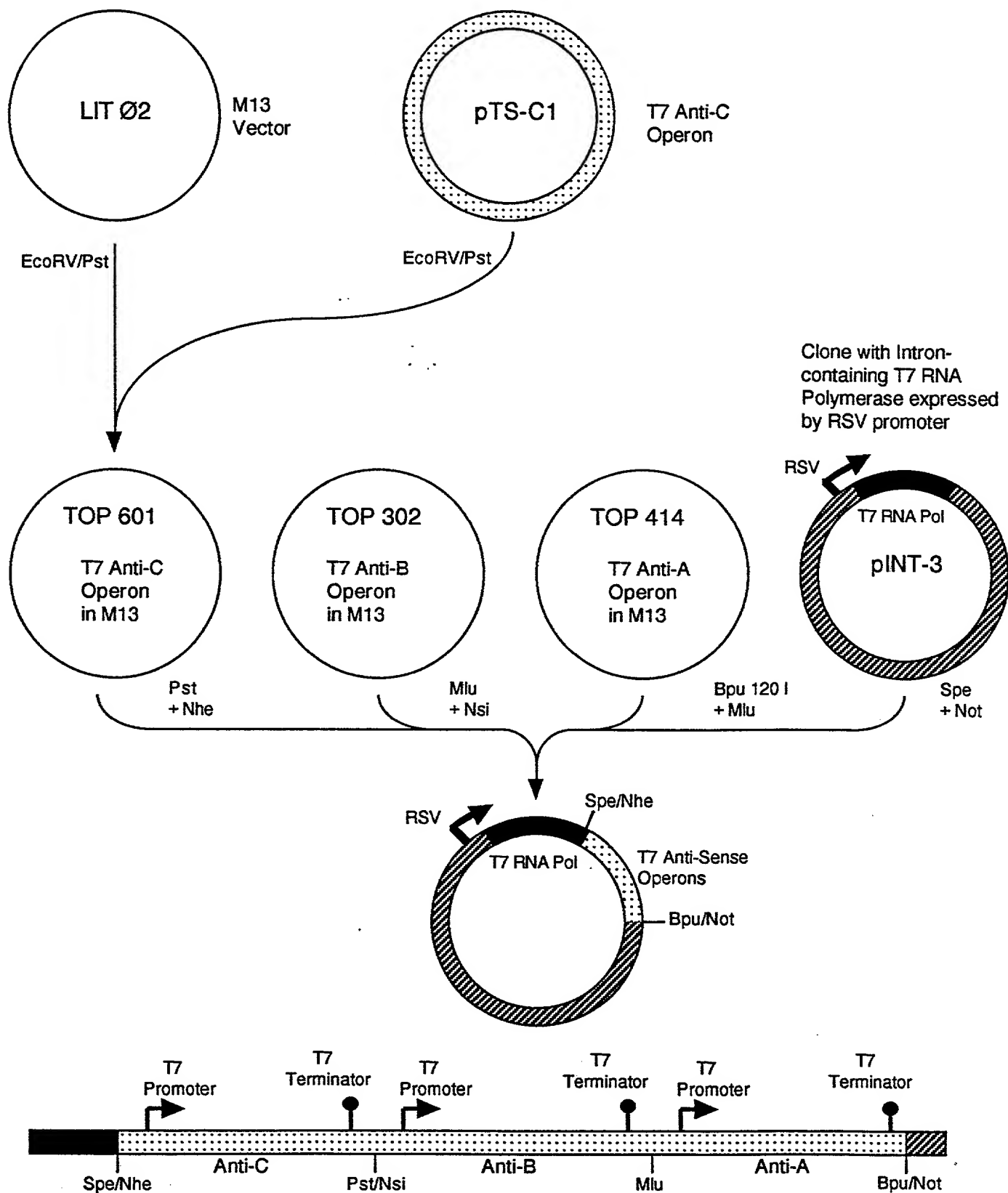


Figure 46

Structures of Triple Operon Constructs
from Figures 44 and 45

**Figure 47**

Construction of Multiple T7 Operons in
Vector coding for T7 RNA Polymerase

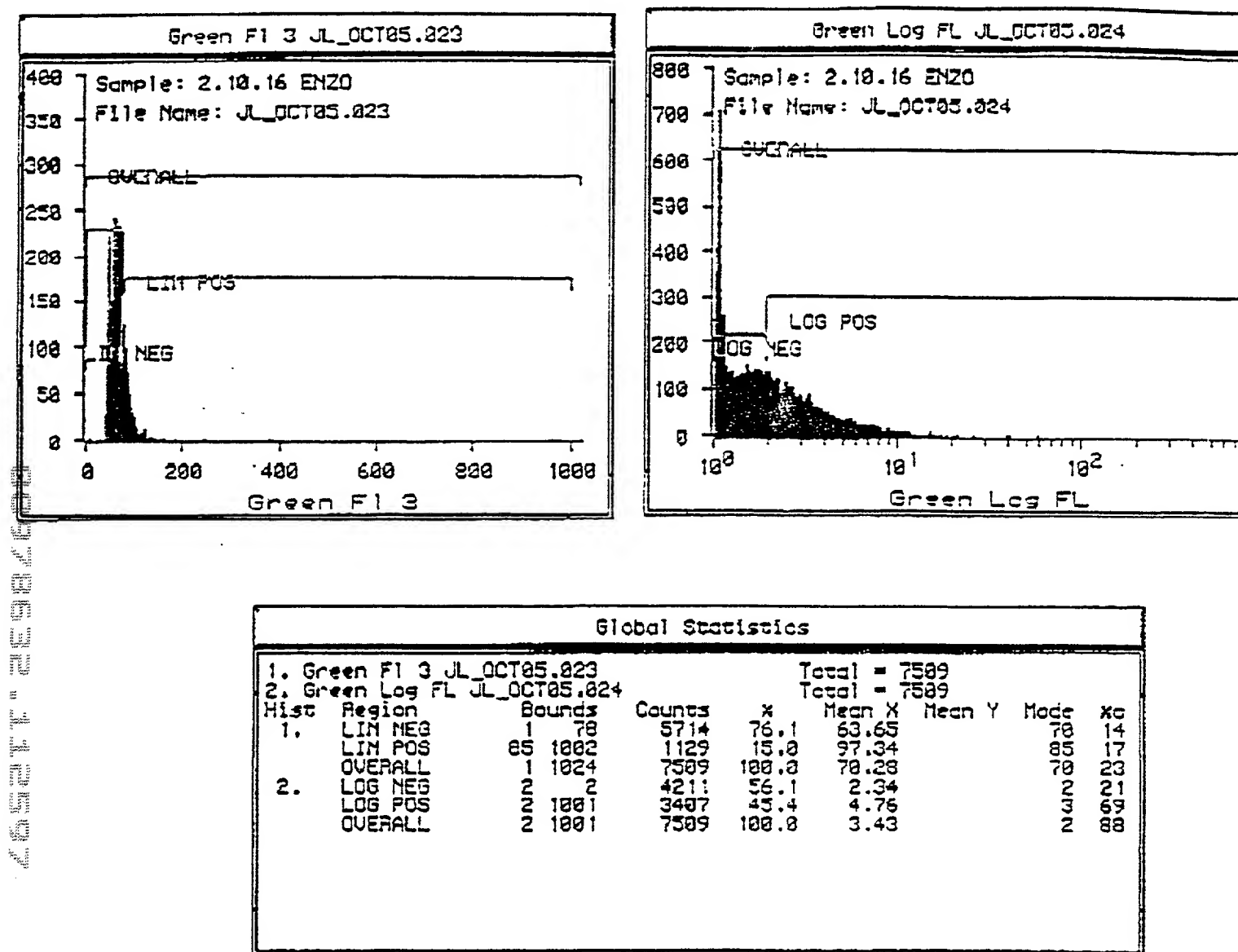


Figure 48

Flow cytometry data measuring binding of
anti-CD4+ antibody to HIV resistant U037 cells



Figure 49

PCR amplification of gag region
indicating absence of HIV in
viral resistant cell line (2.10.16)
after challenge

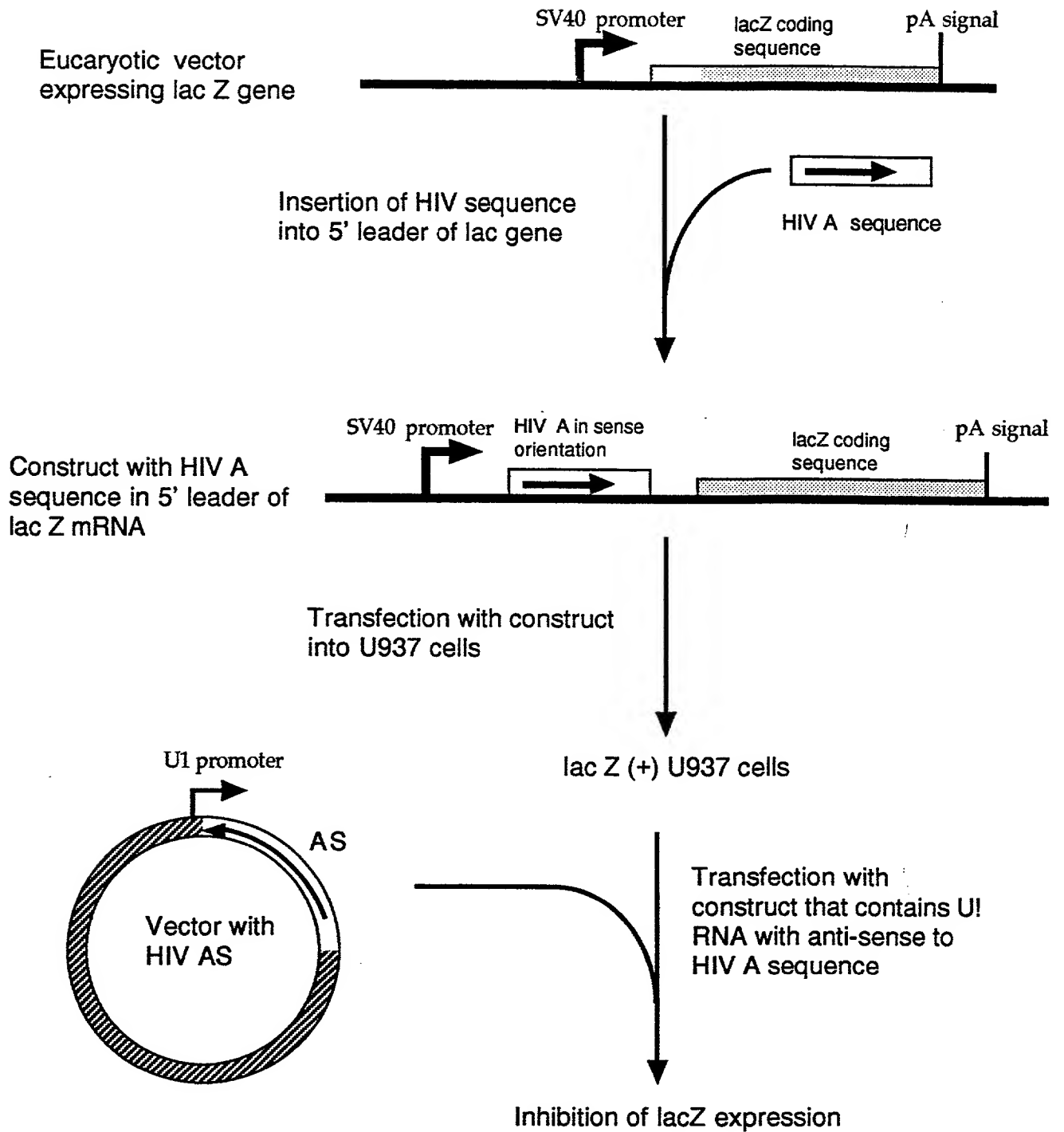


Figure 50

Clone with target-lacZ fusion will have reduced expression of lacZ after transfection by HIV Anti-sense construct

Enzyme activity as expressed by A ₄₂₀ readings in extracts prepared from			
	2.5 x 10 ⁴ cells	5 x 10 ⁴ cells	1.0 x 10 ⁵ cells
U 937 [untransfected]	0.018	0.023	0.034
U 937 [HIV A clone]	0.154	0.277	0.566
U937 [HIV A / Anti-A]	0.010	0.017	0.027
U 937 [HIV A/Anti-ABC]	0.013	0.021	0.035
U 937 [HIV A / Null DNA]	0.120	0.212	0.337

[B] Expression of Beta-galactosidase activity by *In situ* assay :

U 937 [untransfected] no blue spots in cells

U 937 [HIV A clone] blue spots in cells

U 937 [HIV A/Anti A] no blue spots in cells

U 937 [HIV A/Anti ABC] no blue spots in cells

U 937 [HIV A / Null DNA] blue spots in cells

Figure 51

Expression of Beta-galactosidase activity
in extracts



**UNITED STATES DEPARTMENT OF COMMERCE
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.
087374, 443	12/13/95	RABBANI	ENZ-53

18N2/0623
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C/O ENZO BIOCHEM, INC
527 MADISON AVE. 9TH FLOOR
NEW YORK NY 10022

EXAMINER
YUCEL, I

ART UNIT	PAPER NUMBER
1805	

DATE MAILED: 06/23/97

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Please find below and/or attached an Office communication concerning this application or proceeding.

Commissioner of Patents and Trademarks

see the attached.

Notice of Abandonment

Application No.

08/574,443

Applicant(s)

Rabbani et al.

Examiner

Remy Yucel

Group Art Unit

1805



This application is abandoned in view of:

- ☒ applicant's failure to timely file a proper response to the Office letter mailed on Nov 25, 1996.
- ☐ A response (with a Certificate of Mailing or Transmission of _____) was received on _____, which is after the expiration of the period for response (including a total extension of time of _____ month(s)) which expired on _____.
- ☐ A proposed response was received on _____, but it does not constitute a proper response to the final rejection.
(A proper response to a final rejection consists only of: a timely filed amendment which places the application in condition for allowance; a Notice of Appeal; or the filing of a continuing application under 37 CFR 1.62 (FWC)).
- ☒ No response has been received.
- ☐ applicant's failure to timely pay the required issue fee within the statutory period of three months from the mailing date of the Notice of Allowance.
- ☐ The issue fee (with a Certificate of Mailing or Transmission of _____) was received on _____.
- ☐ The submitted issue fee of \$ _____ is insufficient. The issue fee required by 37 CFR 1.18 is \$ _____.
- ☐ The issue fee has not been received.
- ☐ applicant's failure to timely file new formal drawings as required in the Notice of Allowability.
- ☐ Proposed new formal drawings (with a Certificate of Mailing or Transmission of _____) were received on _____.
- ☐ The proposed new formal drawings filed _____ are not acceptable.
- ☐ No proposed new formal drawings have been received.
- ☐ the express abandonment under 37 CFR 1.62(g) in favor of the FWC application filed on _____.
- ☐ the letter of express abandonment which is signed by the attorney or agent of record, the assignee of the entire interest, or all of the applicants.
- ☐ the letter of express abandonment which is signed by an attorney or agent (acting in a representative capacity under 37 CFR 1.34(a)) upon the filing of a continuing application.
- ☐ the decision by the Board of Patent Appeals and Interferences rendered on _____ and because the period for seeking court review of the decision has expired and there are no allowed claims.
- ☐ the reason(s) below:

GEORGE C. ELLIOTT
SUPERVISORY PATENT EXAMINER
GROUP 1800

DECLARATION FOR PATENT APPLICATION

Docket No. **ENZ-53**

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

**NOVEL PROPERTY EFFECTING AND/OR PROPERTY EXHIBITING
COMPOSITIONS FOR THERAPEUTIC AND DIAGNOSTIC USES**

the specification of which

(check one) is attached hereto.

 X was filed on **December 15, 1995**, as U.S. Patent Application Serial No. 08/574,443
and was amended on , 1994 (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)

Priority Claimed

<u> </u> (Number)	<u> </u> (Country)	<u> </u> (Day/Month/Year Filed)	<u> </u> Yes	<u> </u> No
<u> </u> (Number)	<u> </u> (Country)	<u> </u> (Day/Month/Year Filed)	<u> </u> Yes	<u> </u> No

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of the application:

<u> </u> (Application Serial No.)	<u> </u> (Filing Date)	<u> </u> (Status-patented, pending, abandoned)
<u> </u> (Application Serial No.)	<u> </u> (Filing Date)	<u> </u> (Status-patented, pending, abandoned)

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

Ronald C. Fedus, Esq., Reg. No. 32,567.

Address all telephone calls to:
Address all correspondence to:

Ronald C. Fedus, Esq., at telephone no. (212)-856-0876
Ronald C. Fedus, Esq., Corporation & Patent Counsel
Enzo Biochem, Inc.
575 Fifth Avenue - 18th Floor
New York, NY 10017

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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First Inventor's signature [Signature] Date 12/28/95
Residence 99 South Clinton Avenue (No. 11D) Bay Shore, New York 11706 Citizenship United States
Post Office Address (Same as above)

Full name of third joint inventor, if any James J. Donegan
Fourth Inventor's signature [Signature] Date 12/28/95
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Post Office Address (Same as above)

Full name of fourth joint inventor, if any Dakai Liu
Fourth Inventor's signature [Signature] Date 12/26/95
Residence 2 Patricia Court, Bethpage, New York 11714 Citizenship Republic of China
Post Office Address (Same as above)

Full name of fifth joint inventor, if any Norman E. Kelker
Second Inventor's signature [Signature] Date December 20, 1995
Residence 343 East 30th Street (No. 2J), New York, New York 10016 Citizenship United States
Post Office Address (Same as above)

Full name of sixth joint inventor, if any Dean L. Engelhardt
Second Inventor's signature [Signature] Date December 20, 1995
Residence 173 Riverside Drive (No. 6D) New York, New York 10025 Citizenship United States
Post Office Address (Same as above)

* * * * *

ASSIGNMENT

In consideration of One Dollar (\$1.00), and other good and valuable consideration, the receipt of which is hereby acknowledged, I, the undersigned,

Elazar Rabbani,
Jannis G. Stavrianopoulos,
James J. Donegan,
Dakai Liu,
Norman E. Kelker, and
Dean L. Engelhardt,

RESIDING AT:

69 Fifth Avenue (No. 19A), New York, New York 10003;
99 South Clinton Avenue (No. 11D), Bay Shore, New York 11706;
210 East Broadway (No. 3G), Long Beach, New York 11561;
2 Patricia Court, Bethpage, New York 11714;
343 East 30th Street (No. 2J), New York, New York 10016; and
173 Riverside Drive (No. 6D), New York, New York 10025; respectively,

Hereby sell, assign and transfer to: Enzo Therapeutics, Inc., a corporation of the State of New York, having a principal place of business at: 60 Executive Boulevard, Farmingdale, New York 11735-4716, its successors, assigns and legal representatives, the entire right, title and interest for the United States and all foreign countries, in and to any and all improvements which are disclosed in the application for United States Letters Patent, which has been executed by the undersigned concurrently herewith, and is entitled:

NOVEL PROPERTY EFFECTING AND/OR PROPERTY EXHIBITING COMPOSITIONS FOR THERAPEUTIC AND DIAGNOSTIC USES

(said application having been filed on December, 15, 1995 and accorded U.S. Patent Application Serial No. 08/574,443)

and in and to said application and all divisional, continuing, substitute, renewal, reissue, and all other applications for Letters Patent which have been or shall be filed in the United States and all foreign countries on any of said improvements; and in and to all original and reissued patents which have been or shall be issued in the United States and all foreign countries on said improvements:

Agree that said Assignee may apply for and receive Letters Patent for said improvements in its own name, and that, when requested, without charge to but at the expense of said Assignee, its successors, assigns and legal representatives, to carry out in good faith the intent and purpose of this assignment, the undersigned will execute all divisional, continuing, substitute, renewal, reissue, and all other patent applications on any and all said improvements; execute all rightful oaths, assignments powers of attorney and other papers, communicate to said Assignee, its successors, assigns, and representatives, all facts known to the undersigned relating to said improvements and the history thereof; and generally do everything possible which said Assignee, its successors, assigns or representatives shall consider desirable for aiding in securing and maintaining proper patent protection for said improvements and for vesting title to said improvements and all applications for patents and all patents on said improvements, in said Assignee, its successors, assigns and legal representatives; and

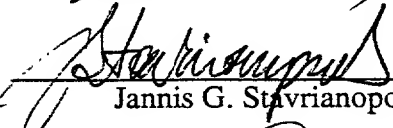
Elazar Rabbani et al.
Serial No. 08/574,443
Filed December 15, 1995 [Assignment]

Covenant with said Assignee, its successors, assigns and legal representatives that no assignment, grant, mortgage, license or other agreement affecting the rights and property herein conveyed has been made to others by the undersigned, and that full right to convey the same as herein expressed is possessed by the undersigned.



Elazar Rabbani

DEC 20 1995
(date)



Jannis G. Stavrianopoulos

12/28/95
(date)



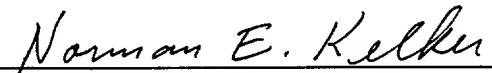
James J. Donegan

Dec 28, 1995
(date)



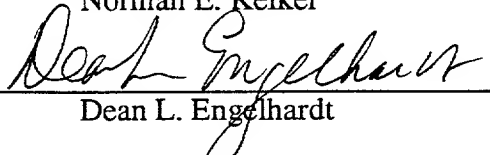
Dakai Liu

Dec. 26, 1995.
(date)



Norman E. Kelker

December 20, 1995
(date)



Dean L. Engelhardt

December 20, 1995
(date)

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s): Elazar Rabbani et al.

Serial No.: 08/574,443

Group Art Unit: Not Yet Known

Filed: December 15, 1995

Examiner: Not Yet Known

For: NOVEL PROPERTY EFFECTING AND/OR PROPERTY EXHIBITING
COMPOSITIONS FOR THERAPEUTIC AND DIAGNOSTIC USES**VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY
STATUS (37 CFR 1.9 (f) and 1.27 (c)) - SMALL BUSINESS CONCERN**

I hereby declare that I am

- ☐ the owner of the small business concern identified below:
- ☒ an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF CONCERN ENZO THERAPEUTICS, INC.ADDRESS OF CONCERN c/o ENZO BIOCHEM, INC.
575 FIFTH AVENUE (18TH FLOOR)
NEW YORK, NEW YORK 10017

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 13 CFR 121.3-18, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees under section 41(a) and (b) of Rule 35, United States Code, in that the number of employees of the concern, including those of its affiliates does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention entitled:

**NOVEL PROPERTY EFFECTING AND/OR PROPERTY EXHIBITING
COMPOSITIONS FOR THERAPEUTIC AND DIAGNOSTIC USES**

by inventor(s)

Elazar Rabbani et al.

described in

- ☐ the specification filed herewith
- ☒ Application Serial No. 08/574,443, filed December 15, 1995
- ☐ Patent No. _____, issued _____

If the rights held by the above identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27).

NAME _____

ADDRESS _____
() Individual () Small Business Concern () Nonprofit

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small business entity is no longer appropriate. (37 CFR 1.28(b)).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statement may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING

BARRY W. WEINER

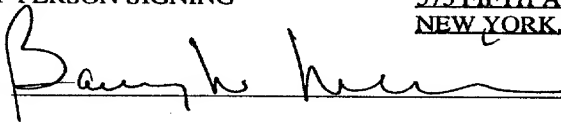
TITLE OF PERSON OTHER THAN OWNER

EXECUTIVE VICE PRESIDENT

ADDRESS OF PERSON SIGNING

575 FIFTH AVENUE (18TH FLOOR)
NEW YORK, NEW YORK 10017

SIGNATURE

DATE MARCH 8, 1996

08978632, 112592